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John Goodpaster
Chair

Paul Shepson

Greg Michalski

Brenda Blacklock

Jay A. Siegel

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Approved by Major Professor(s): John Goodpaster

Approved by: E. C. Long 11/14/2013
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MONITORING, CHARACTERIZING, AND PREVENTING MICROBIAL DEGRADATION OF IGNITABLE LIQUIDS ON SOIL

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With much love and thanks for my amazing family and friends. My sister, Lee, and my church family, you supported and encouraged me throughout my studies. Pastor Barry and Ruth Curtis you pushed me to pursue my education, so without you I would not be where I am today. Last but not least, I thank God for giving me the strength and determination to pursue and complete my education.

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LIST OF ABBREVIATIONS

ASTM	American Society for Testing and Materials
BSU	Ball State University
Cd	cadmium
Cr	chromium
CS_2	carbon disulfide
DA	discriminant analysis
DFLEX	diffusive flammable liquid extraction
DTPA	diethylenetriaminepentaacetic acid
EIC	extracted ion chromatogram
FDA	Federal Drug Administration
Fe	iron
FEMA	Federal Emergency Management Agency
GC-MS	gas chromatography-mass spectrometry
*gyr*B	gyrase subunit B
HPD	heavy petroleum distillate
ILR	ignitable liquid residue
IPA	isopropanol
K	potassium

MPD	medium petroleum distillate
MS	mass spectral
MSDS	materials safety data sheet
n-	normal
N	total nitrogen
NCFS	National Center for Forensic Science
NIST	National Institute of Standards and Technology
P	phosphorus
Pb	lead
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PEG	polyethylene glycol
SPC	standard plate count
SPME	solid phase microextraction
SRN	standard reference number
T/SWGFEX	Technical and Scientific Working Group for Fire and Explosions
TIC	total ion chromatogram
TOC	total organic carbon
TSA	tryptic soy agar
TSB	tryptic soy broth
UCF	University of Central Florida
Zn	zinc

ABSTRACT

Turner, Dee A. Ph.D., Purdue University, December 2013. Monitoring, Characterizing, and Preventing Microbial Degradation of Ignitable Liquids on Soil. Major Professor: John Goodpaster.

Organic-rich substrates such as soil provide an excellent carbon source for bacteria. However, hydrocarbons such as those found in various ignitable liquids can also serve as a source of carbon to support bacterial growth. This is problematic for fire debris analysis as samples may be stored at room temperature for extended periods before they are analyzed due to case backlog. As a result, selective loss of key components due to bacterial metabolism can make identifying and classifying ignitable liquid residues by their chemical composition and boiling point range very difficult. The ultimate goal of this project is to preserve ignitable liquid residues against microbial degradation as efficiently and quickly as possible.

Field and laboratory studies were conducted to monitor microbial degradation of gasoline and other ignitable liquids in soil samples. In addition to monitoring degradation in potting soil, as a worst case scenario, the effect of soil type and season were also studied. The effect of microbial action was also compared to the effect of weathering by evaporation (under nitrogen in the laboratory and by the passive headspace analysis of the glass fragments from the incendiary devices in the field studies). All studies showed

that microbial degradation resulted in the significant loss of n-alkanes and lesser substituted alkylbenzenes predominantly and quickly, while more highly substituted alkanes and aromatics were not significantly affected. Additionally, the residential soil during the fall season showed the most significant loss of these compounds over the course of 30 days.

To combat this problem, a chemical solution is to be immediately applied to the samples as they are collected. Various household and commercial products were tested for their efficacy at low concentrations to eliminate all living bacteria in the soil. Triclosan (2% (w/v) in NaOH) proved to be the most effective at preserving ignitable liquid residues for at least 30 days.

CHAPTER 1. INTRODUCTION

1.1 Review of Fire Debris Analysis

Microbial degradation of ignitable liquids poses a significant threat to the identification and classification of their residues in highly organic matrices such as soil. Microorganisms present in the soil quickly alter chromatographic profiles of ignitable liquids by selectively metabolizing hydrocarbons in the ignitable liquid residues. Therefore the ultimate purpose of the work described herein is to prevent microbial degradation in soil samples by application of an antimicrobial solution to soil samples containing ignitable liquids.

In many criminal cases where there has been a fire, an ignitable liquid may have been involved. The vast majority of ignitable liquids are hydrocarbon-based fuels. The most common one, owing to availability and cost, is gasoline. Other common consumer products may also be used, and they are classified by chemical composition and boiling point. These other product classes include petroleum distillates (e.g., lighter fluid, charcoal starter fluid, diesel), isoparaffinics (e.g., paint thinner), aromatics (e.g., degreasers), naphthenic paraffinics (e.g., lamp oil), n-alkanes (e.g., candle oil), de-aromatized distillates (e.g., camping fuel), oxygenated solvents (e.g., ketones), and miscellaneous products such as turpentine [1].

The task of the forensic fire debris examiner in these cases is two-fold: remove the ignitable liquid residues from the matrix and determine the type of ignitable liquid that is present. There are a number of validated methods for concentration or isolation of liquid residues from fire debris. These include steam distillation [2], solvent extraction [3], headspace sampling [4], and passive and dynamic headspace concentration [5, 6].

Even though steam distillation is still an acceptable method by American Society for Testing and Materials (ASTM) standards, it is really only useful when large amounts of the ignitable liquid are present in the sample [18]. Solvent extraction is also an approved ASTM method; however, other methods such as passive headspace concentration have been designed since to be far more sensitive. Solvent extraction is a useful method when samples are very small, when the ignitable liquid contains compounds with very high boiling points, or when the matrix is unsuitable for extraction of the ignitable liquid by other methods [18]. Steam distillation and solvent extraction are traditional concentration methods that offer major disadvantages including the amount of time and work involved compared to their effectiveness, as well as the loss of key compounds and the background interference that is sometimes generated [18].

1.2 Extraction Techniques

Modern methods of extraction include dynamic and passive headspace concentration methods. These isolation techniques allow the ignitable liquids to volatilize and concentrate onto an adsorption media that can be then be subsequently desorbed for analysis . Dynamic headspace concentration involves the use of an inert gas to continually purge the headspace of the sample allowing the ignitable liquid to be

completely extracted from the matrix and collected onto an adsorbent material such as activated charcoal or Tenax [7]. Thermal desorption (in the case of Tenax) or solvent extraction (for activated charcoal) is then used to release the ignitable liquid from the adsorbent material [7]. The drawback of using dynamic headspace concentration is the phenomenon known as breakthrough, which results in the unrecoverable loss of the ignitable liquid residue. This loss occurs due to the flow rate being too high, a saturation of the adsorbent not allowing further compounds to be adsorbed, or a long-lasting draft through the tube causing dilution of the adsorbed compounds [7].

The method used in most forensic laboratories is passive headspace concentration, so this was the chosen method for this research [15-17]. Passive headspace concentration is a much simpler method for sample extraction and is also non-destructive, making it the preferred method for sample extraction [7]. As with dynamic headspace concentration, the ignitable liquid is vaporized and collected onto an adsorbent material, generally a porous polymer or carbon. The ignitable liquid can be subsequently desorbed off the strip thermally or extracted using a solvent. The difference between passive and dynamic headspace concentration is that for passive headspace concentration, an inert gas is not used to force the ignitable liquid out of the headspace and onto the adsorbent material, as it is in dynamic headspace concentration. Instead the adsorbent material is suspended into the headspace as the sample is heated. Also, this method utilizes a closed system whereas dynamic headspace concentration does not. An advantage of passive headspace concentration is that it allows the sample to be stored for re-analysis at a later time, if needed. Suitable solvents for passive headspace concentration should have the ability to extract the compounds in the ignitable liquid from the adsorbent material. Suitable

solvents include: carbon disulfide, methanol, dichloromethane, diethyl ether, and pentane [7]. Carbon disulfide has been the solvent of choice since it is much more efficient and has a higher solubility for the ignitable liquids than the other solvents, however, because of its cost, flammability, and toxicity, it has been replaced with other solvents that are not quite as effective, but are safer [7]. Pentane has replaced carbon disulfide in many laboratories as it is suitable for extracting most ignitable liquid residues from the charcoal strip. It is also much safer than carbon disulfide and does not largely interfere with the analysis of the ignitable liquid residue. The major drawback to the use of pentane is that it does not extract oxygenated compounds as well as carbon disulfide does and it has a similar boiling point to some of the lower boiling oxygenated compounds, which can be lost with pentane during GC-MS analysis.

Solid phase microextraction (SPME) is a new technique that uses an adsorbent coated silica fiber instead of a carbon strip [7]. The fiber is encased in a hollow syringe, so the fiber can be exposed to the sample and then retracted for analysis. SPME is versatile as it can be used in a headspace method, direct method, or partial headspace method; however, the recovery of each of these methods varies. SPME is not currently a widely accepted method for the analysis of fire debris, as it tends to be selective according to the properties of the compounds in the ignitable liquid, it is not possible to re-analyze samples at a later time, and the difficulty of using automated sampling [7]. The method for identification of the residue in forensic science laboratories is universally gas chromatography, generally using silicone columns and mass spectrometry detection [8].

1.3 Microbial Degradation of Ignitable Liquids

The phenomenon whereby microorganisms metabolize components of petroleum has been well studied over the past several decades. For example, indigenous microbial communities found in oil fields are extremely diverse and include numerous species of bacteria [9]. Numerous trends and observations that have been noted in the literature [10] regarding the degradation of particular classes of hydrocarbons in petroleum can be summarized as follows:

- C_6 through C_{15} n-alkanes are the most readily degraded components of petroleum
- Typically, the first sign of biodegradation is loss of n-akanes in the C_{10} through C_{13} range
- Aromatic hydrocarbons are more resistant than aliphatic hydrocarbons to degradation
- Cyclic and branched chain alkanes are more resistant than straight chain hydrocarbons
- Resistance to degradation increases with the degree of substitution in isoalkanes, alkylcyclohexanes, alkylcyclopentanes and alkylbenzenes.
- Resistance to degradation also depends on substitution effects (e.g., 3-methyl alkanes > 4-methylalkanes > 2-methylalkanes)
- Adjacent methyl groups (e.g., 1,1-dimethylcyclohexane and 1,2,3-trimethylbenzene) also increase resistance to biodegradation

- Degradation in heavier aliphatic hydrocarbons tends to occur in a sequence whereby n-alkanes are removed first followed by acyclic isoprenoid alkanes (e.g., phytane)

Not surprisingly, the propensity of bacteria and other microorganisms to consume petroleum products can have a detrimental effect on the identification of ignitable liquids, particularly in highly organic samples such as soils. D.C. Mann and W.R. Gresham of the Washington State Highway Patrol Crime Laboratory first explored this phenomenon in the context of fire debris analysis in 1990 [11]. Using garden soil spiked with gasoline, this study demonstrated that degradation occurred rapidly unless the soil was either thoroughly sterilized prior to introduction of gasoline or the gasoline/soil samples were stored at -5 ^{o}C. For unsterilized samples stored at room temperature, the degradation process was characterized by a loss of substituted benzenes and all n-paraffinic compounds within a few days. The isoparaffinic compounds, however, were not affected. As a result of these findings, the authors stated that all soil submitted to the laboratory would henceforth be kept in a freezer until analysis is completed.

Soon after the publication of this paper, K.P. Kirkbride and co-workers isolated two species of bacteria (*Pseudomonas putida* and *Pseudomonas fluorescens biovarIII*) from soil samples that had generated an anomalous chromatographic pattern [12]. The ability of these bacteria to degrade gasoline and petroleum naphtha was evaluated *in vitro* and the two species were found to be complementary in their action, in that *P. putida* consumed aromatic portions of the fuels while *P. fluorescens biovarIII* consumed the aliphatic portion. Finally, the authors offered recommendations for avoiding microbial

degradation such as storing samples at reduced temperature (as per [11]) or adding a non-volatile bactericide to the fire debris (although this was not explored). Alternatively, if microbial degradation is seen, the authors recommended demonstrating the presence of bacteria by culturing the fire debris samples and screening for species that are known to degrade petroleum and produce anomalous chromatographic profiles.

Cherry and co-workers analyzed gasoline and a medium and a heavy petroleum distillate on three different types of soil using a dynamic heated headspace technique and gas chromatography [13]. A control sample of each ignitable liquid was placed on paper toweling to simulate weathering. Samples were analyzed the same day, and after 1, 2, and 3 weeks. Degradation was prominent in the soil that was darker and damper than the other two soil types. Furthermore, significant degradation was not seen until 2 weeks had past. It was determined that microbial degradation did occur among the n-alkanes in both of the petroleum distillates. Microbial degradation occurred among the aromatics in gasoline, but to a lesser extent than the degradation among the n-alkanes in the petroleum distillates.

More recently, D. Chalmers and co-workers repeated the work by Mann and Gresham using GC/MS technology as well as evaluating the effect of microbes on gasoline, a medium petroleum distillate (MPD) and a heavy petroleum distillate (HPD) [14]. Both rural and garden soils were used as substrates. In addition, Diffusive Flammable Liquid Extraction (DFLEX®) charcoal strips, which are physically isolated from the debris by permeable membranes, were used. Although no longer commercially available, DFLEX strips were intended to be added to the debris when the container is sealed. Ignitable liquid residues were then adsorbed onto the charcoal strip until the

evidence was opened, then the strip was desorbed with a suitable solvent. Significant degradation of n-alkanes and mono-substituted aromatics was noted in all samples, albeit after a longer time period (7 to 14 days) than was seen in some other works.

1.4 Chemometric Analysis of Fire Debris

The complex nature of gasoline and other ignitable liquids makes statistical approaches attractive for the data analysis of fire debris samples. A review by Sandercock discusses the many statistical approaches that have been applied to the analysis of various neat and weathered ignitable liquids [15] and one of the most common statistical methods being Principal Component Analysis (PCA) [16-19]. Overall, these studies have been focused on the chemical fingerprinting of ignitable liquids, with a particular emphasis on discriminating and identifying different ignitable liquids in varying stages of weathering. More recent studies have used various statistical approaches such as Discriminant Analysis (DA) and target factor analysis to distinguish various ignitable liquids in fire debris samples that contain contributions from various matrices [20, 21].

1.5 Preventing Degradation of Ignitable Liquids

Of the significant number of fire debris samples the Indiana State Police (ISP) laboratory receives, about 10% of them contain soil. As the samples often sit for extended periods of time, microbial degradation of the ignitable liquid residue is apparent.

Therefore it became our goal to not only monitor degradation in all different types of ignitable liquids, but also develop a method for preserving fire debris evidence.

Many methods have been suggested and/or are used to reduce or eliminate microbial activity in soil samples, although most have not been suggested for use in fire debris. These methods include moist heat sterilization (autoclaving), dry heat sterilization, γ-irradiation, microwaves, gaseous chemicals, and the addition of chemical solutions such as mercuric chloride and sodium azide [22-28]. Autoclaving soil entails heating it at about 120°C at 1.1 atm for about 30 minutes to an hour depending on sample size [22, 27]. Autoclaving also requires the use of a thin layer, no more than 2cm thick so that the steam can penetrate the soil. Autoclaving thicker layers can result in the survival of some bacteria even after repeating the process 2-3 times. Nowak and Wronkowska investigated the treatment of soil with antibiotics in addition to autoclaving and found that antibiotics do not increase the efficiency of soil sterilization [29]. Dry heat sterilization entails heating the sample for at least 24 hours at a much higher temperature than autoclaving (200°C) [22]. A thin layer of soil must also be used for dry heat sterilization. The use of γ-irradiation to sterilize soil samples uses a Co-60 source to irradiate moisture-adjusted soil in sealed polyethylene bags at 2.5Mrad at 2Mrad/hr [22]. Higher doses (e.g. 4Mrad) can be used for smaller bacteria such as *Pseudomonas fluorescens* [22]. The use of microwave radiation has been studied for sterilizing soil samples at 2450MHz [22, 27]. The bacterial cells absorb heat, resulting in a temperature increase that is high enough to kill the bacteria. Since water also absorbs heat, microwave radiation works better for moist samples compared to dry samples [22]. Gaseous chemicals such as chloroform, methyl bromide, ethylene oxide and propylene oxide have also been used via fumigation

of the moist soil inside a desiccator [22]. Chemicals such as mercuric chloride and sodium azide have also been used to inhibit bacterial activity in soil samples [22]. A mercuric chloride solution of sufficient concentration such that 500mg mercuric chloride per kilogram of soil is achieved is reportedly enough to inhibit microbial activity. Sodium azide prepared to a final concentration of between 1 and 10% is typically enough to inhibit microbial activity in soil samples [22]. Alphei and Scheu showed that soil sterility was achieved by subjecting soil samples to gamma irradiation, propylene oxide fumigation, methyl bromide fumigation (at a concentration of 0.04mol/L) and by autoclaving [27]. Furthermore, their work showed that methyl bromide fumigation at 0.01mol/L, microwave radiation, and chloroform fumigation were all unsuccessful in killing microorganisms in the soil. However, methyl bromide depletes the ozone layer significantly and therefore production has been phased out [28]. A review of acute gamma irradiation by McNamara and co-workers suggested that while most species of soil bacteria were eliminated between 15 and 25kGy, a higher dose may be necessary to achieve soil sterility [26]. Additionally, soils that contain higher levels of organic matter may require even higher doses of gamma irradiation as these soils shield the bacteria against the gamma irradiation [26]. Yamamoto and co-workers showed that some bacterial populations, particularly gram-positive bacteria, were able to recover from chloropicrin and methyl bromide fumigations while the soil sterilization method had a greater impact on all bacteria [25].

Ozone has also been known to kill bacteria and other microorganisms as it is a powerful oxidizing agent [28]. Ozone generated on site requires the use of either a dielectric barrier discharge, pulsed discharge, corona discharge, or surface discharge [28].

Ebihara and co-workers developed a system for generating ozone that is injected into the soil to sterilize it using coaxial dielectric barrier discharge, which efficiently produced a high concentration of ozone [28].

Many of the suggested methods are not feasible for the preservation of fire debris evidence. Fire debris samples not only typically sit for weeks prior to analysis, but fire investigators do not transport fire debris samples to the laboratory for analysis immediately. Therefore samples containing soil are often significantly degraded before analysis and possibly even before they are received by the laboratory. This renders any method that requires application in the laboratory ineffective. Additionally, packaging samples in a cooled storage container (such as dry ice) for transport back to the laboratory followed by refrigerated or freezer storage is also not practical in crime laboratories. This is due to not only cost for such storage, but also because it relies on fire investigators to transport the samples back to the laboratory in a timely fashion, which does not occur consistently. Instead, a method that can be applied in the field should be utilized, which would kill all bacteria in the sample so that fire debris samples are no longer subject to microbial degradation and therefore will be preserved until the time of analysis regardless of the lapsed time between sample collection and sample analysis.

1.6 Research Goals

The purpose of this research is three fold. First, this work seeks to monitor microbial degradation over time in order to extend what is known about the effects of degradation on ignitable liquids in qualitative and semi-quantitative terms. Attempts have

been made to identify signature profiles for the various accelerants such that degraded samples could still be identified. In particular, representatives from each major class of ignitable liquid (as defined by ASTM) will be included as microbial degradation of several ignitable liquid classes has yet to be reported. In addition to initial studies of microbial degradation in potting soil over 7 days presented in Chapter 2, a more detailed discussion of microbial degradation of exemplars from each class compared to weathering is presented in Chapter 3. These liquids were provided by the University of Central Florida as a collaborative project to add to a ignitable liquids database. Semi-quantitative comparisons of samples from our initial studies have also been made to elucidate specific chemical classes that are more susceptible to degradation in ignitable liquids that may be present in evidentiary samples.

Secondly, this work seeks to characterize the effects of weathering and microbial degradation on gasoline samples using Principal Components Analysis (PCA) in order to elucidate relationships between variables that may be less obvious. In particular, the effect of the two processes on the chromatographic profile of gasoline will be discussed so that those compounds that are least vulnerable to either weathering or degradation can be identified. Additionally, in a collaborative study with Ball State University, microbial degradation of gasoline in 3 soil types (collected by our collaborators) was monitored over 4 seasons. PCA was also applied to elucidate trends among these soil types and seasons.

Thirdly, this work seeks to prevent microbial degradation via the application of a chemical treatment that can be applied in the field immediately upon collection of the evidence, which would preserve ignitable liquid residues for later analysis. Many of the

previously mentioned methods are not ideal for use in the field or do not kill all bacteria in the soil. A chemical treatment would be a more efficient means for treatment of soil samples in the field. An ideal antimicrobial solution for the use in fire debris samples should be water soluble, non-volatile (e.g., a polar compound with a high molecular weight), relatively non-toxic to humans, does not interfere with sample analysis, and is easy to use by non-scientists. Triclosan is a known antimicrobial used in hand soaps, lotions and other household products. In this work, we investigated its use as well as other household chemicals as antimicrobial solutions for treatment of soil samples.

CHAPTER 2. MONITORING DEGRADATION

2.1 Introduction

Soil, like other materials rich in organic matter, provides a rich medium for bacteria to grow. Some bacteria can also metabolize hydrocarbons such as those found in gasoline and other ignitable liquids. Research in the area of bioremediation has demonstrated that bacteria can metabolize hydrocarbons found in crude oil. However, previous to this work, the phenomenon of microbial degradation has not been well studied in fire debris samples. Kirkbride's work has shown that there are two strains of bacteria commonly found in soil which are capable of metabolizing hydrocarbons found in gasoline [12]. His and other work has shown that the n-alkanes and the lesser substituted aromatics are most susceptible to microbial degradation [12, 14, 30]. However, only degradation of the more common ignitable liquids has been reported thus far. According to the Indiana State Police Forensic Science Laboratory in Indianapolis, approximately 10% of fire debris samples are found to be degraded. Our work seeks to not only show microbial degradation of common ignitable liquids, but also in liquids that had not been reported previously. In addition, this work seeks to provide a more quantitative approach to the analysis of microbial degradation of ignitable liquids.

2.2 Materials and Methods

2.2.1 Chemicals

The ignitable liquids: gasoline (87 octane), odorless lighter fluid (Isoparaffinic), charcoal starter fluid (medium petroleum distillate), and kerosene and fuel oil #2, dyed (heavy petroleum distillates) were provided by the Indiana State Police Laboratory, Microanalysis Unit. An ATSM E1618-97 test mixture containing hexane, toluene, octane, p-xylene, 3-ethyltoluene, 2-ethyltoluene, 1,2,4-trimethylbenzene, decane, dodecane, tetradecane, hexadecane, octadecane and eicosane 0.05% (v/v) each in methylene chloride was obtained from Restek. Other chemicals include dichloromethane, pentane, *m*-xylene and *p*-xylene obtained from Fisher Scientific. The substrate used was Hyponex® brand potting soil obtained from Wal-Mart. Activated charcoal strips were obtained from Albrayco Technologies. Crochet Nylon thread was obtained from Wal-Mart. #1 size paperclips were obtained from Office Depot. Quart-sized paint cans were obtained from Lab Safety Supply.

2.2.2 Procedure

20µL of the ignitable liquid was spiked onto a thin layer of potting soil (40 – 90 grams) inside a quart-sized paint can. The can was then either immediately extracted as described below or sealed. After storage at room temperature for either two or seven days, the can was then re-opened and a third (~7x9 mm^2) of a carbon strip was suspended on a pre-baked paper clip into the headspace of the can. The can was then resealed and baked in the oven at 85°C for 4 hours. Upon cooling the can to room temperature, the carbon strip was placed in a test tube and the ignitable liquid was extracted by adding 300µL of pentane and vortexing for 1 minute. A soil control for each time point was also prepared.

A standard solution of each ignitable liquid was also prepared (0.1 and 1% v/v in pentane) for retention time comparisons.

2.2.3 Instrumental Method

All data was acquired using an Agilent 6890 Gas Chromatograph with an Agilent 5975 Mass Spectrometer. A Gerstel MPS2 autosampler was also used. The GC was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 microns). The carrier gas was helium with a flow rate of 1mL/min. The method utilized an inlet temperature of 250°C, 1 µL injection volume, and a 20:1 split ratio. The default oven temperature program started at 40°C for 3 minutes, ramped to 280°C at 10°C/min. and held for 3 minutes. The MS parameters included a 3 minute solvent delay and a scan range of m/z 40-300. This method is similar to the method used by the Indiana State Police laboratory for the analysis of fire debris evidence.

2.2.4 Data Analysis

Each component was identified based on comparison of its retention time and mass spectrum to the ASTM standard and the NIST mass spectral database. In addition, extracted ion chromatograms (EIC) were generated for either the n-alkanes (m/z 57, 71, 85, 99) or the aromatics (m/z 91, 105, 119), according to ASTM standards [18, 19]**.** The extracted ion profile allows the analyst to filter out signals from pyrolysis products or other contaminants that can interfere with the ability to identify the ignitable liquid [19]. The EIC peak areas for each compound of interest were summed to obtain an overall area. The relative area of each component was then calculated by dividing the summed EIC peak areas by the total EIC peak areas of all components (n-alkane or aromatic). The area of each of the compounds in the ignitable liquids was determined by integrating the

extracted ion profiles for the alkanes (m/z 57, 71, 85, 99) and for gasoline, the aromatics (m/z 91, 105, 119) as per the method used by the Microanalysis Unit of the Indiana State Police Laboratory. Any peaks for compounds of importance that were not integrated automatically using the software were manually integrated. As long as the peak could be visualized, it was integrated in order to obtain an accurate area. A peak area of zero was reported for compounds that did not have a visible peak in the extracted ion profile. Compounds with a very small peak that could be manually integrated in the total ion chromatogram (TIC) but were not visible in the extracted ion profile were reported as having zero area. The peak areas from the TIC could be used for non-complex ignitable liquids, in which the compounds are completely resolved. However, the ignitable liquids studied thus far are complex and therefore not all the compounds are completely resolved from one another. Therefore, to ensure that peak area of one compound was for only that compound, the extracted ion profiles were used. The summed peak areas were compared over time, before degradation and after degradation occurred. Note that an internal standard for ignitable liquid residues on complex matrices such as soil had not yet been established. Such a standard needs to be one that will not only be similar to the compounds in the ignitable liquid, but also not subject to microbial degradation.

2.3 Results and Discussion

Gasoline contains n-alkane and aromatic compounds as well as branched and cycloalkane compounds. This complex mixture can then be subject to perturbations such as weathering or microbial degradation. The term weathering is used to describe the effects of evaporation of an ignitable liquid; however, there are other causes of weathering other than just simple evaporation. When an ignitable liquid is subjected to

weathering from an environmental perspective, the ignitable liquid suffers from heat and light exposure as well as changes in the headspace due to turbulence [7]. Weathering results in a skewed chromatographic profile toward the heavier components in the ignitable liquid. Therefore, an initial comparison of fresh gasoline and weathered gasoline is made in order to contrast the effects of weathering (which should depend largely on boiling point) to that of microbial degradation (which demonstrates compound selectivity). The total ion chromatograms (TICs) for samples of fresh and weathered gasoline are shown in Figure 2-1. These and all chromatograms presented in our work were normalized to the most abundant peak in the chromatogram. This normalization is common practice in crime laboratories as the question of amount of sample is not relevant. The legal question pertaining to fire debris is whether or not an ignitable liquid residue is present. Additionally, the passive headspace extraction technique does not lend itself to quantitative analysis as the amount of ignitable liquid residue present is greater than the activated charcoal strip can adsorb. Therefore only ratios of compounds found in ignitable liquid residues are compared to that of a standard ignitable liquid. Visual inspection of the chromatograms from weathering versus microbial degradation shows that weathering manifests itself as the loss of those components that elute around 5 minutes or less, while the other components remain relatively unaffected. Quantitative comparisons support this interpretation. Table 2-1 contains relative peak area difference data for the n-alkane and aromatic components of gasoline in the fresh versus weathered samples. Consistent with visual examination of the TICs in Figure 2-1, weathering is characterized by a dramatic negative difference in the most volatile component of each class (i.e., heptane and toluene). Little to no difference is seen among the heaviest

components (i.e., dodecane and 1,2,4-trimethylbenzene). Lastly, if one considers the target compounds according to their structural class (alkane or aromatic), the relative proportion of peak area due to alkanes is higher in the fresh sample (7.6%) versus the weathered sample (3.1%).

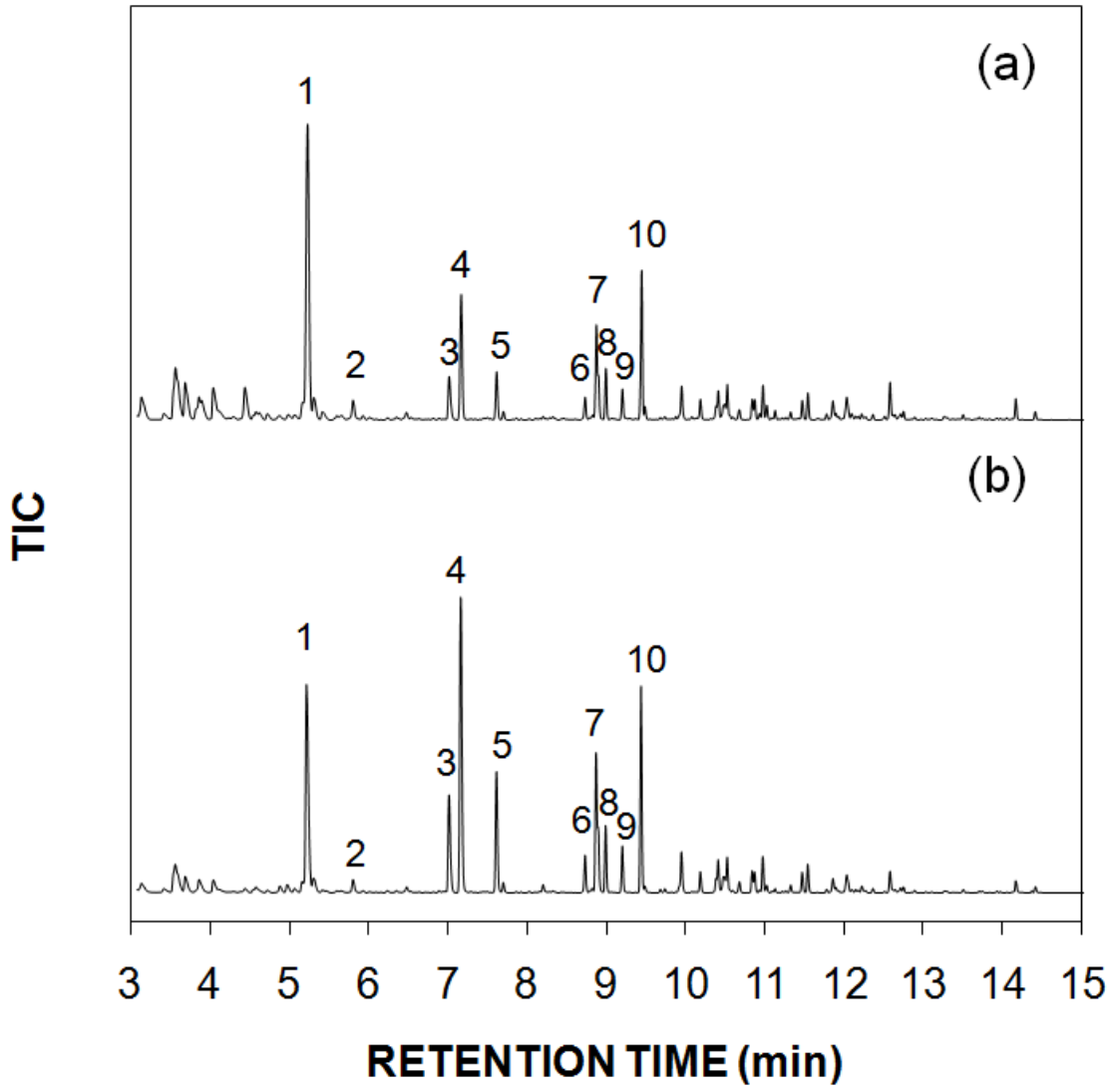


Figure 2-1 Total ion chromatograms of (a) fresh and (b) slightly weathered gasoline standards diluted 0.67% v/v in pentane. Peaks: (1) toluene, (2) n-C_8, (3) ethylbenzene, (4) *m*- and *p*-xylene, (5) o-xylene (6) propylbenzene, (7) 3-ethyl toluene, (8) 1,3,5-trimethylbenzene, (9) 2-ethyl toluene (10) 1,2,4-trimethylbenzene.

Table 2-1 Comparison of the difference in relative EIC peak areas for n-alkanes (m/z 57, 71, 95, 99) in weathered gasoline versus degraded gasoline. Boiling points were obtained from the Materials Safety Data Sheets (MSDSs) for each compound.

		Difference in Relative Peak Areas (%)	
Component	**Boiling Point (^{o}C)**	**Weathered**	**Degraded**
Heptane	98	-14.2	24.7
Octane	125-127	7.4	40.8
Nonane	151	9.6	-15.5
Decane	172-174	-1.4	-21.6
Undecane	196	-0.8	-17.3
Dodecane	216	-0.6	-11.1

In contrast, Figure 2-2 shows the effect of exposing the weathered gasoline sample to either “living” or autoclaved (sterile) soil for two days. In this case, the default GC oven program was altered to optimize resolution of the lighter components (4 ^{o}/min ramp to 90 ^{o}C, then 20 ^{o}/min to 300 ^{o}C). In the soil exposed to “living” soil, the lighter alkanes do not show a relative decrease as was seen with weathering. In fact, heavier alkanes in the ranges of C_9 through C_{12} show significant losses, consistent with microbial degradation. This carbon number range is suspected to be optimal for microbial degradation in terms of enthalpy of reaction and water solubility [10]. The greatest losses among the aromatic compounds occurred among the mono-substituted benzenes (i.e., toluene, ethylbenzene and propylbenzene) as well as 1,2,4-trimethylbenzene. The latter compound is particularly crucial for the identification of gasoline in fire debris. The loss of 1,2,4-trimethylbenzene (peak 10) can be visually detected by noting the reversal in the

ratio of its peak height to that of 3-ethyltoluene (peak 7). Taken together, these results verify that the selective loss of the components in gasoline originate from microbial degradation and not from weathering or poor recovery. While it is known that potting soil is typically sterilized, bacteria that benefit plant growth, such as diazotrophs can be found in potting soil. Culturing experiments (discussed in Chapter 6) showed that potting soil contained living bacteria. While no reduction of select hydrocarbons was observed in the autoclaved soil, potting soil that was not autoclaved showed signs of microbial degradation (Figure 2-2).

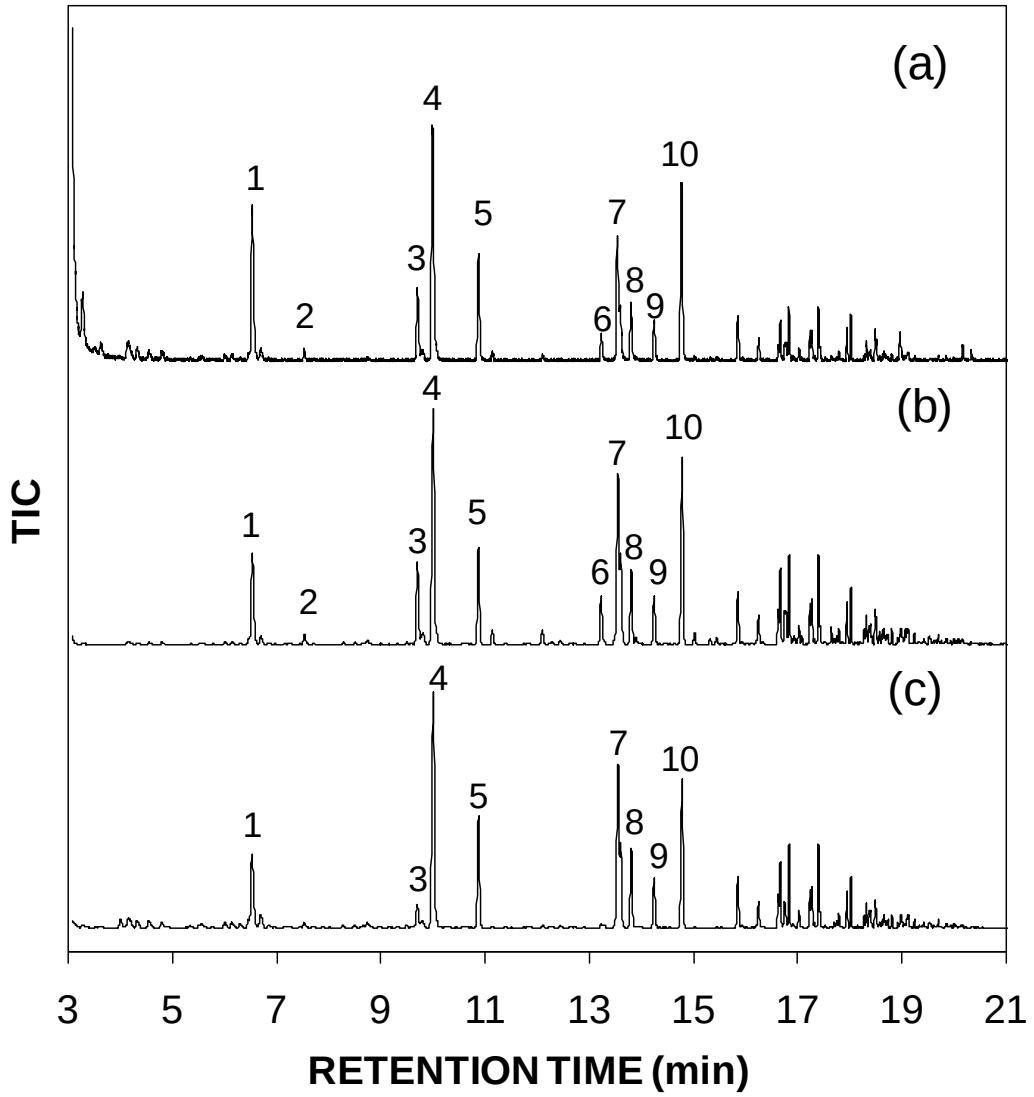


Figure 2-2 Total ion chromatogram of gasoline (a) diluted 1% v/v in pentane, (b) recovered from autoclaved soil after two days, and (c) recovered from soil after two days. Peaks: (1) toluene, (2) n-C_8, (3) ethylbenzene, (4) *m*- and *p*-xylene, (5) *o*-xylene (6) propylbenzene, (7) 3-ethyl toluene, (8) 1,3,5-trimethylbenzene, (9) 2-ethyltoluene (10) 1,2,4-trimethylbenzene.

The degradation of gasoline was studied over time periods extending up to seven days, the results of which are shown in Figure 2-3. Table 2-2 and 2-3 contain relative peak areas for the aliphatic and aromatic components of the sample following immediate recovery (0 days) and after two days on the soil. After two days, n-alkanes such as octane

(Peak 2) and decane (Peak 11) largely disappear from the TIC and the mono-substituted benzenes also show significant decreases. In addition, the peak height ratio of 3-ethyltoluene and 1,2,4-trimethylbenzene reverses. After seven days, there are no peaks in the TIC that are readily attributable to gasoline. The peaks seen at ~ 4 minutes correspond to volatile short-chain aldehydes that are detected in the headspace of the soil under normal circumstances.

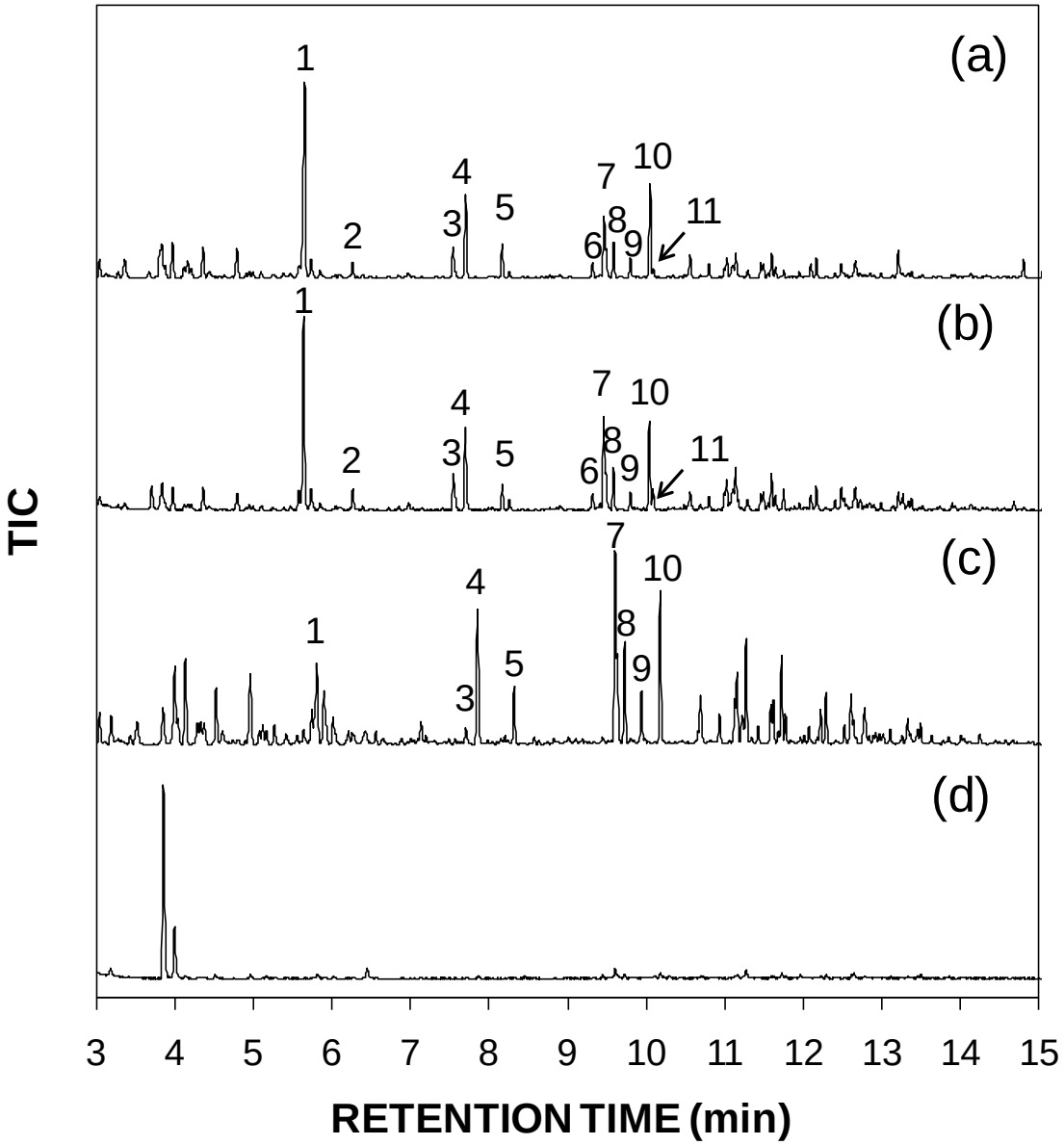


Figure 2-3 Total ion chromatogram of gasoline: (a) standard diluted 0.67% v/v in pentane, (b) after 0 days on soil, (c) after 2 days on soil, and (d) after 7 days on soil. Peaks: (1) toluene, (2) n-C_8, (3) ethyl benzene, (4) *m*- and *p*-xylene, (5) *o*-xylene (6) propylbenzene, (7) 3-ethyltoluene, (8) 1,3,5-trimethylbenzene, (9) 2-ethyltoluene (10) 1,2,4-trimethylbenzene, (11) n-C_{10}.

Table 2-2 Comparison of the difference in relative EIC peak areas for aromatics (m/z 91, 105, 119) in weathered gasoline and degraded gasoline. Boiling points were obtained from the Materials Safety Data Sheets (MSDSs) for each compound.

		Difference in Relative Peak Areas (%)	
Component	**Boiling Point (^{o}C)**	**Weathered**	**Degraded**
Toluene	111	-21.8	-0.4
Ethylbenzene	136	3.0	-5.5
m- and *p*-xylene	138 - 139	10.7	4.7
o-xylene	143-145	4.4	3.1
Propylbenzene	158	0.5	-4.7
3-ethyltoluene	158-159	2.7	2.6
1,3,5-trimethylbenzene	163-165	0.0	0.9
2-ethyltoluene	164-165	0.3	0.5
1,2,4-trimethylbenzene	168	0.0	-1.1

Table 2-3 Comparison of the relative EIC peak areas (m/z 57, 71, 95, 99) for n-alkanes in a gasoline sample recovered from soil immediately (0 days) and recovered from soil after two days.

	Relative Peak Areas (%)		
Component	**After 0 days**	**After 2 days**	**Difference**
Heptane	17.5	88.4	70.9
Octane	17.7	11.6	-6.1
Nonane	9.6	0.0	-9.6
Decane	19.8	0.0	-19.8
Undecane	20.4	0.0	-20.4
Dodecane	15.0	0.0	-15.0

The potential risk of misclassifying an ignitable liquid due to microbial degradation is particularly relevant when dealing with petroleum distillates. This stems from the fact that petroleum distillates are comprised of branched and n-alkane compounds, the latter of which is more susceptible to microbiological attack. For example, charcoal starter fluid is a medium petroleum distillate, with n-alkanes ranging from C_9 to C_{11} (Figure 2-4). The n-alkanes are degraded rapidly while the branched alkanes remain unchanged. After 7 days very little of the n-alkanes are present and the resultant profile resembles that of an isoparaffin. In contrast, odorless lighter fluid is comprised solely of branched alkanes, which shows little to no degradation even after seven days on soil (Figure 2-5).

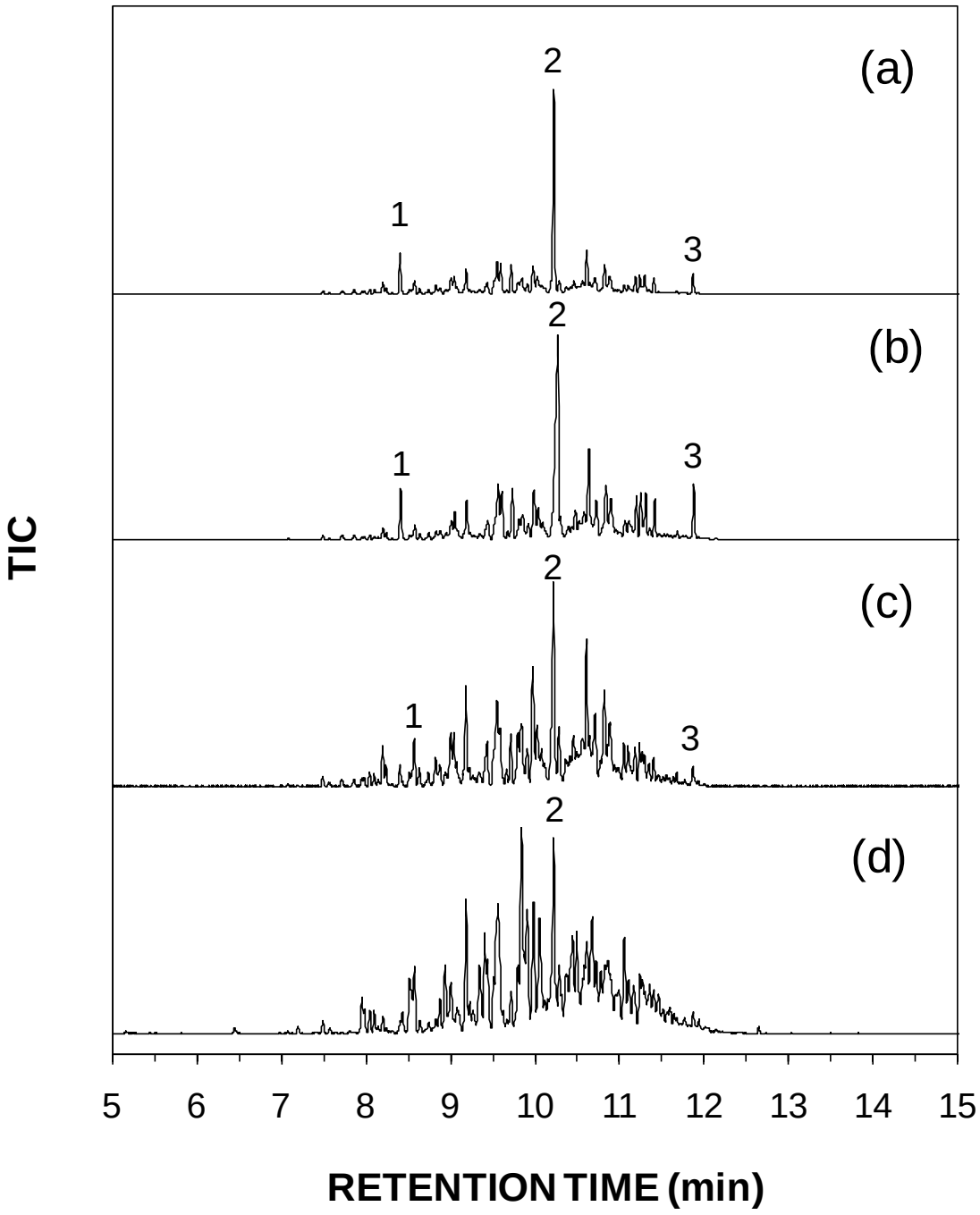


Figure 2-4 Total ion chromatogram for a charcoal starter, a medium petroleum distillate: (a) standard diluted 0.1% v/v in pentane, (b) after 0 days on soil, (c) after 2 days on soil, (d) after 7 days on soil. Peaks: (1) n-C_9, (2) n-C_{10}, (3) n-C_{11}.

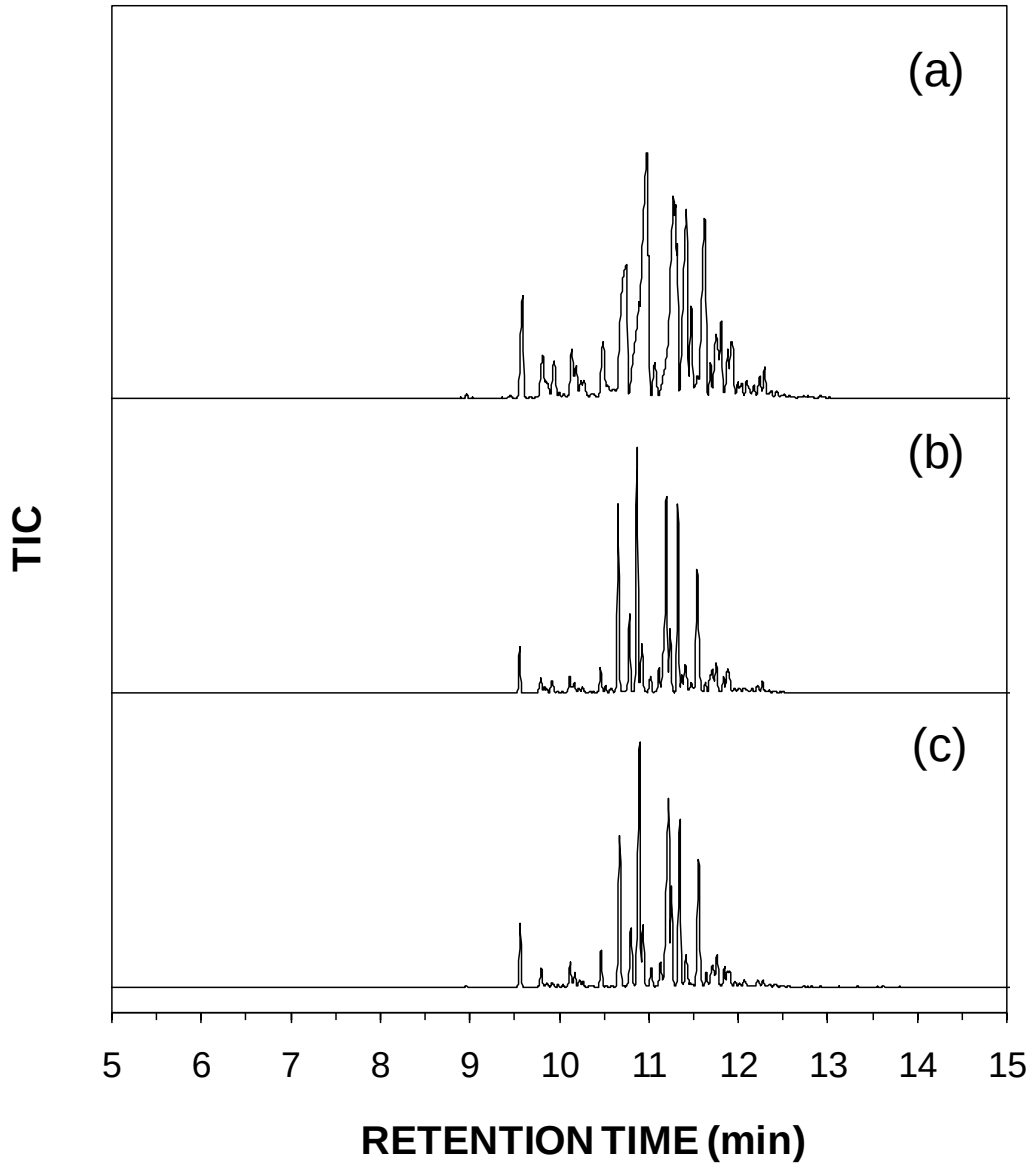


Figure 2-5 Total ion chromatogram of an odorless lighter fluid, a medium-heavy isoparaffin: (a) standard diluted 1% v/v in pentane, (b) after 0 days on soil, and (c) after 7 days on soil. Peaks: All branched alkanes between C_{11} and C_{15}.

A heavy petroleum distillate such as kerosene consists of a mixture of branched and n-alkanes (Figure 2-6). In this sample, the characteristic Gaussian-like distribution of n-alkanes rapidly begins to change even after 2 days. After 7 days, all that remains is an

unresolved alkane envelope comprised mainly of branched alkanes. Analysis of the relative peak areas (Table 2-4) shows that the loss of n-alkanes occurred in both the lighter ($C_{10} - C_{11}$) and heavier ($C_{15} - C_{16}$) n-alkanes. Fuel oil was the least volatile of the petroleum distillates studied in this work. As a consequence, there is a discernible difference between the chromatographic profile of the liquid standard and the residue recovered immediately from the soil. This difference may be attributable to poor recovery of the heavy petroleum fraction (i.e., $C_{20} - C_{22}$) due to partitioning into the organic matter in the soil. The degradation pattern for this ignitable liquid was consistent with the trends discussed above for petroleum, in that the n-alkanes from C_{11} to C_{15} are degraded more than the n-alkanes from C_{16} to C_{20} (Table 2-5).

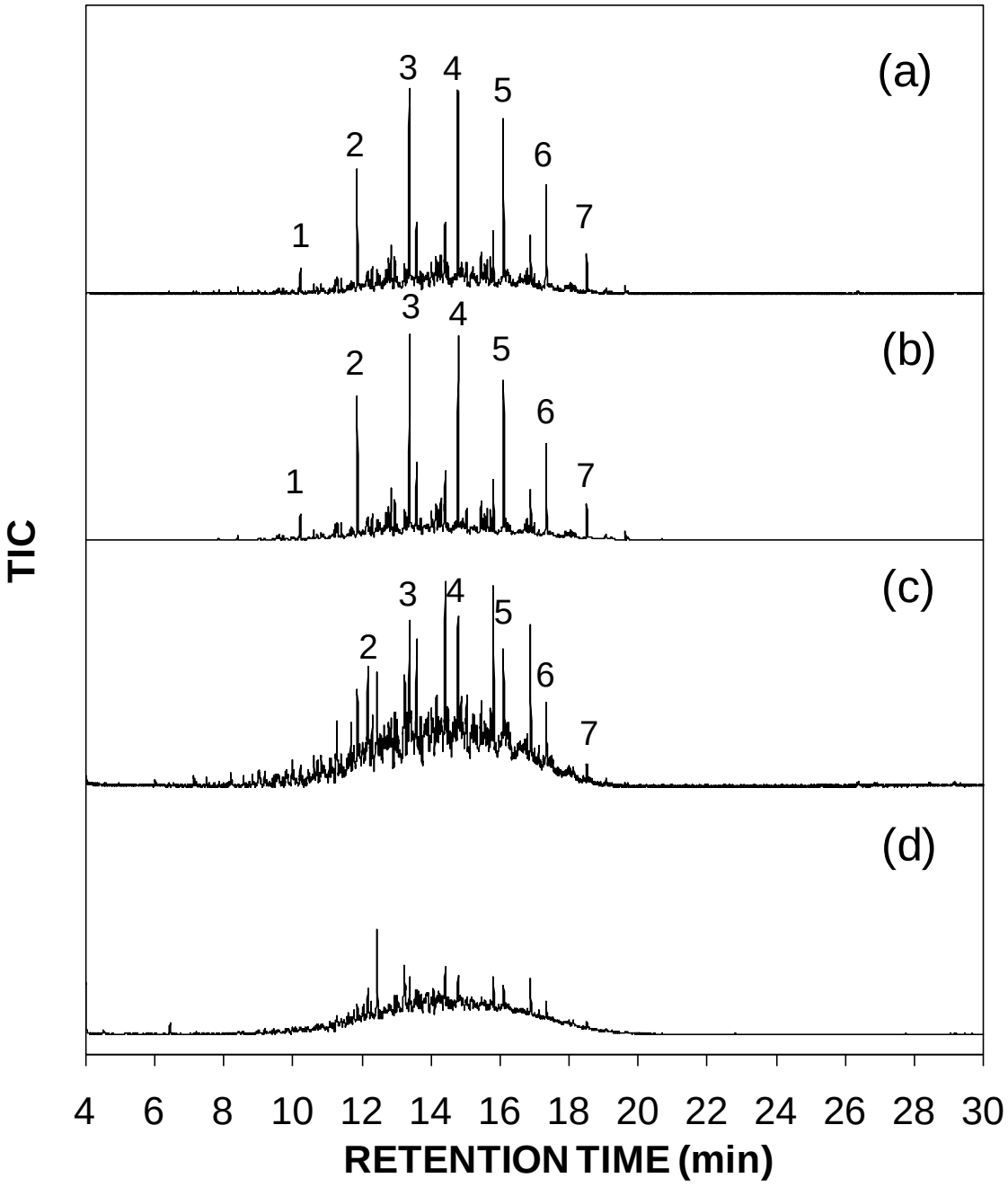


Figure 2-6 Kerosene, a heavy petroleum distillate: (a) standard diluted 0.1% v/v in pentane, (b) after 0 days on soil, (c) after 2 days on soil, and (d) after 7 days on soil. Peaks: (1) n-C_{10}, (2) n-C_{11}, (3) n-C_{12}, (4) n-C_{13}, (5) n-C_{14}, (6) n-C_{15}, (7) n-C_{16}.

Table 2-4 Comparison of the relative EIC peak areas (m/z 57, 71, 95, 99) for n-alkanes in a kerosene sample recovered from soil immediately (0 days) and recovered from soil after two days.

	Relative Peak Areas (%)		
Component	**After 0 days**	**After 2 days**	**Difference**
Decane	2.7	0.0	-2.7
Undecane	15.4	11.6	-3.8
Dodecane	24.9	27.1	2.2
Tridecane	23.9	27.1	3.2
Tetradecane	17.5	20.4	2.9
Pentadecane	11.5	11.4	-0.1
Hexadecane	4.1	2.3	-1.8

Table 2-5 Comparison of the relative EIC peak areas (m/z 57, 71, 95, 99) for n-alkanes in a fuel oil sample recovered from soil immediately (0 days) and recovered from soil after two days.

	Relative Peak Areas (%)		
Component	**After 0 days**	**After 2 days**	**Difference**
Undecane	0.5	0.0	-0.5
Dodecane	3.7	0.0	-3.7
Tridecane	9.8	2.6	-7.2
Tetradecane	18.0	8.5	-9.5
Pentadecane	25.2	19.2	-6.0
Hexadecane	22.6	23.2	0.6
Heptadecane	14.7	22.9	8.2
Octadecane	4.5	13.6	9.1
Nonadecane	0.9	7.4	6.5
Eicosane	0.0	2.5	2.5

De-aromatized petroleum distillates are chemically very similar to other petroleum distillates, the only significant difference being the removal of aromatic compounds [1, 7]. De-aromatized distillates contain an abundance of normal alkanes, as well as a significant, but less abundant, amount of branched and cyclic alkanes [1, 7, 31]. One such ignitable liquid is Tiki Torch fuel, depicted in Figure 2-7. By visual inspection, the loss of undecane (peak 1) and dodecane (peak 3) is apparent, while an increase in relative peak abundance of the branched alkanes is noted. In addition, an increase in relative peak abundance of 2-methyldecahydronaphthalene (peak 2) was also observed. Differentiating between de-aromatized distillates and petroleum distillates can be quite difficult since they are so chemically similar. However, aromatic components also are degraded by microorganisms [12, 30, 32, 33]. Therefore degraded samples of a de-aromatized distillate and a petroleum distillate will look even more similar than their non-degraded counterparts, making it even more difficult to distinguish between these ignitable liquids.

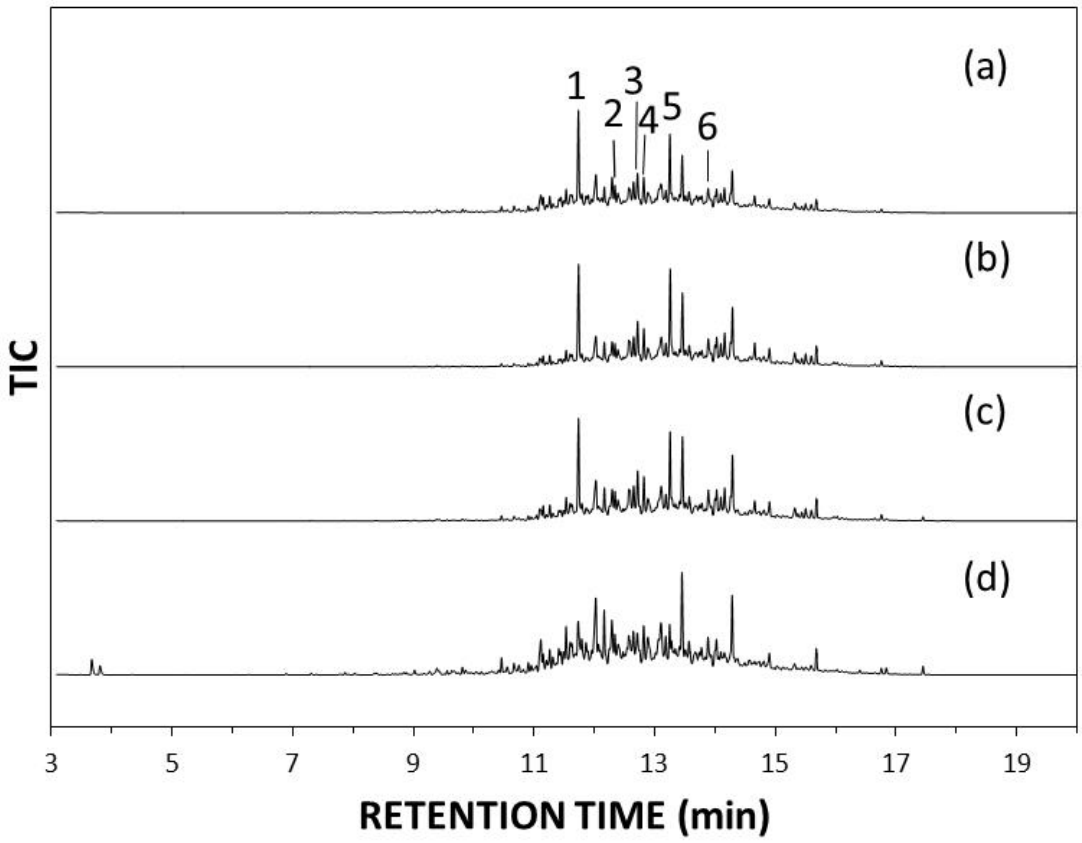


Figure 2-7 Total ion chromatogram of a tiki torch fuel, a de-aromatized distillate: (a) standard, 0.1% (v/v), (b) Day 0, (c) Day 2, (d) Day 7. Peaks: (1) n-C_{11}, (2) pentylcyclohexane, (3) 2-methylundecane, (4) 3-methylundecane, (5) n-C_{12}, and (6) hexylcyclohexane.

A naphthenic-paraffinic product is comprised of predominately branched alkanes and cyclic alkanes, so there are not significant amounts of normal alkanes or aromatics [1, 7]. Therefore, as shown in Figure 2-8, even after 7 days no significant changes in relative peak abundance were observed. However, as stated by Huang and Larter [34] even though alkyl substitution reduces susceptibility to degradation, the position of the methyl group is also a factor. For example, a 3-methylalkane is more resistant to biodegradation than a 4-methylalkane, which is more resistant than a 2-methylalkane. This is seen in

Figure 2-8 with a change in the peak ratio of 2-methylundecane (peak 2) and 3-methylundecane (peak 3). Also, both of these compounds have a reduced peak area relative to 7-methyltridecane (peak 6). Furthermore, the peak area of 2-methyl- and 3-methylundecane is reduced compared to the peak area for the more substituted 2,6-dimethylundecane (peak 4).

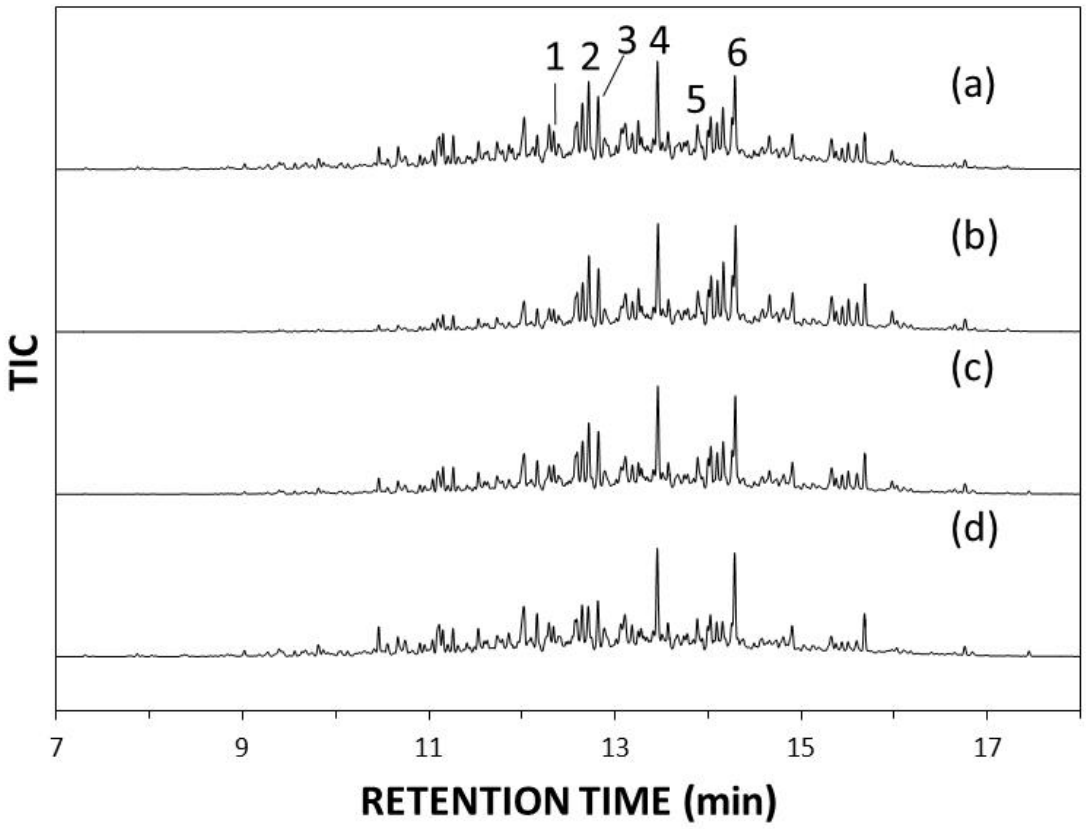


Figure 2-8 Total ion chromatogram of a lamp oil, a naphthenic-paraffinic liquid: (a) Standard, 0.1% (v/v), (b) Day 0, (c) Day 2, (d) Day 7. Peaks: (1) pentylcyclohexane, (2) 2-methylundecane, (3) 3-methylundecane, (4) 2,6-dimethylundecane, (5) hexylcyclohexane, and (6) 7-methyltridecane.

Ignitable liquids that do not fit into any of the other classifications are classified as a miscellaneous liquid [1, 7]. One such ignitable liquid is turpentine, a natural product

derived from pine wood, comprised of various terpenes [7]. Biotransformation of terpenes has been well studied. Bacteria such as pseudomonas have been known to transform limonene into oxygenated derivatives such as carveol, carvone, perillic acid, and limonene-1,2-diol [35]. Limonene has also been studied as the sole carbon source for different types of bacteria [36]. Bicas and co-workers determined that of the 238 strains of bacteria tested, 70 grew well where limonene was the only carbon source, many of which are gram positive bacteria, such as Pseudomonas [36]. Bicas and co-workers also reported biodegradation of α-pinene and β-pinene in both strains of Pseudomonas [37]. In addition, P. fluorescens degraded limonene [37]. Terpenes have also been studied as a stimulator for microbial degradation of PCBs [38, 39]. Our results are consistent with these studies in that there was a clear decrease in relative peak area of limonene (peak 6), *o*-cymene (peak 5), and *β*-pinene (peak 3) upon exposure to soil (Figure 2-9).

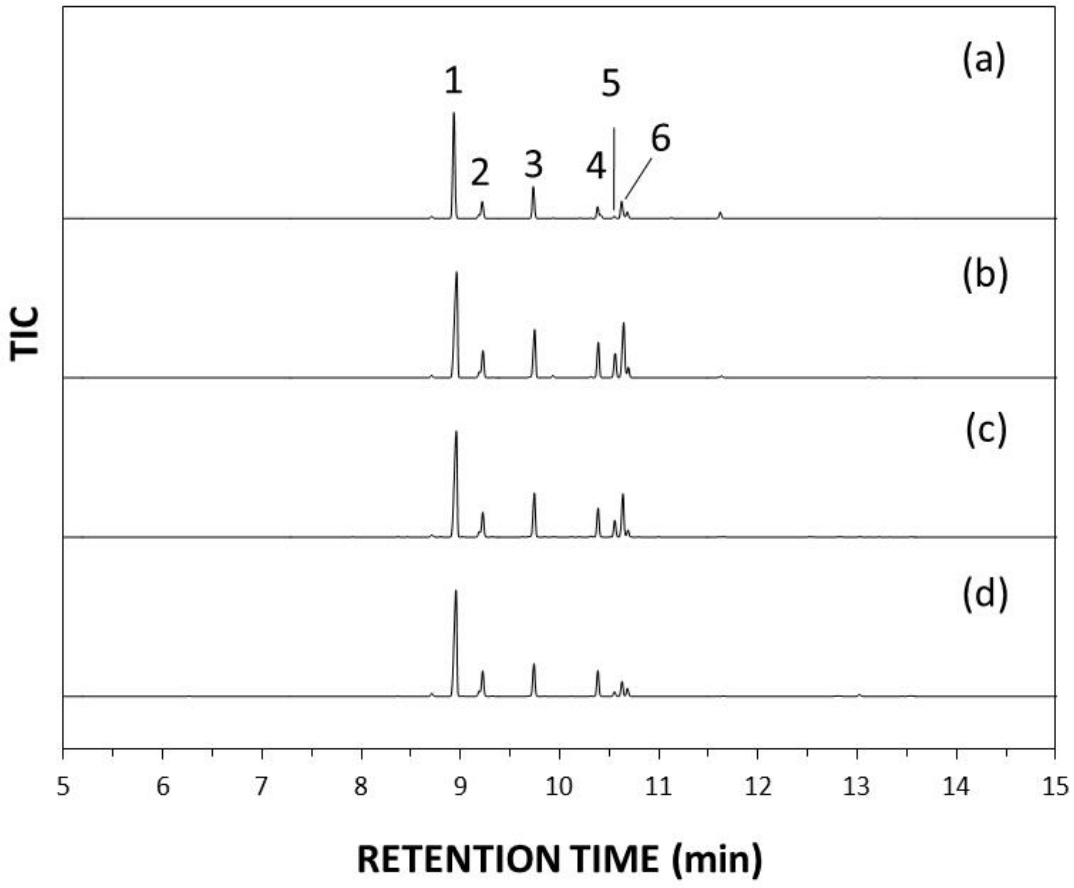


Figure 2-9 Total ion chromatogram of turpentine, a miscellaneous liquid: (a) standard, 0.1% (v/v), (b) Day 0, (c) Day 2, and (d) Day 7. Peaks: (1) *α*-pinene, (2) camphene, (3) *β*-pinene, (4) 1,4-cineole, (5) *o*-cymene, and (6) limonene.

2.4 Conclusions

The ability of microbes to degrade petroleum products is a well-known phenomenon and one that can be harnessed for environment remediation. However, this is a decidedly unfavorable process when it occurs in fire debris awaiting analysis. Several authors have noted some of the effects of microbial degradation on ignitable liquids in soils and this work seeks to broaden what is known to other classes of ignitable liquids as well as provide semi-quantitative estimates of the effects of degradation on chromatographic

profiles. In particular, the loss of straight chain alkanes, particularly in the range of C_9 – C_{16}, was evident in all samples. Key compounds in gasoline were also degraded, with mono-substituted benzenes such as toluene, ethylbenzene and propylbenzene being the most vulnerable. 1,2,4-trimethylbenzene was also degraded relative to surrounding peaks in the chromatogram. Additionally, a loss of the normal alkanes was observed in the tiki torch fuel, making it difficult to distinguish from an isoparaffinic product. However, branched and cyclic alkanes are also subject to microbial degradation, particularly 2- and 3-methylalkanes, which was demonstrated by the loss of 2-methylundecane and 3-methylundecane in the lamp oil. Furthermore, branched alkanes that are more highly substituted and those with methyl groups at a higher position on the alkyl chain are more resistant to degradation. As shown with the lamp oil, 2,6-dimethylundecane and 7-methyltridecane were more resistant to degradation than 2-methylundecane and 3-methylundecane. Terpenes also provide a carbon source for microbial degradation, particularly limonene, *o*-cymene, and *β*-pinene, found in turpentine. Overall, this represents a new type of perturbation on ignitable liquid residues that may influence the interpretation of chromatographic data from fire debris evidence.

CHAPTER 3. UNIVERSITY OF CENTRAL FLORIDA DEGRADATION STUDIES

3.1 Introduction

Microbial degradation is a phenomenon that affects ignitable liquid residues found in highly organic matrices like soil. Currently, there is not a database that contains samples of all classes of ignitable liquids that have been subjected to microbial degradation. This will be a valuable tool for fire debris analysts in the identification and classification of ignitable liquid residues. The purpose of the work presented here is a collaborative project in conjunction with Dr. Michael Sigman and co-workers at the University of Central Florida (UCF) to monitor degradation over time for samples of ignitable liquids in each class as determined by the American Society for Testing and Materials (ASTM). The data generated from this work was then added to the database collection containing fresh and weathered ignitable liquids as well as fire debris substrates, developed by the National Center for Forensic Science (NCFS) and the Technical and Scientific Working Group for Fire and Explosions (T/SWGFEX).

3.2 Materials and Methods

3.2.1 Materials

The ignitable liquids were supplied by Dr. Michael Sigman's laboratory (Table 3-1). The substrate used was Hyponex® brand potting soil obtained from K-Mart. Activated charcoal strips were obtained from Albrayco Technologies. Quart-sized paint cans were

obtained from Best Containers. Pentane was purchased from Fisher Scientific and carbon disulfide was purchased from Alfa Aesar.

Table 3-1 Ignitable liquids provided by UCF for this study, along with the sample reference number (SRN) and ASTM classification.

SRN	Liquid	Class
059	Adhesive Remover	Aromatic
005	ShellSol A100	Aromatic
052	Ortho Malathion 50 Plus Insect Spray Conc.	Aromatic
073	Whitaker Ar-Sol 15 (Aromatic 150)	Aromatic
284	Exxon Aromatic 100	Aromatic
116	Gasoline	Gasoline
105	Phillips 66 Unleaded Regular Gasoline	Gasoline
258	Chevron Regular Unleaded Gasoline	Gasoline
259	Chevron Plus Unleaded Gasoline	Gasoline
NewGas1	Texaco Regular Unleaded Gasoline	Gasoline
NewGas2	Murphy USA Regular Gasoline	Gasoline
NewGas3	Shell Gasoline	Gasoline
E-85	E-85 Gasoline	Gasoline
014	STP Fuel Injector/Carburetor Cleaner	Heavy Petroleum Distillate
043	Chevron Techron Concentrate	Heavy Petroleum Distillate
050	Chevron Low Sulfur Diesel Fuel 2	Heavy Petroleum Distillate
020	Penske Fuel Injector/Carburetor Cleaner	Heavy Petroleum Distillate
120	Isopar C	Isoparaffinic
087	Isopar E	Light Isoparaffinic
035	Zippo Premium Lighter Fluid	Light Petroleum Distillate
008	Shell Rubber Solvent 332	Light Petroleum Distillate
033	Ronsonol Lighter Fluid	Light Petroleum Distillate
119	Isopar H	Medium Isoparaffinic
012	Shellsol Odorless Mineral Spirits	Medium Isoparaffinic
089	Isopar M	Medium Isoparaffinic

021	Mineral Spirits/Paint Thinner	Medium Petroleum Distillate
046	Pro-Gard Fuel Injector PLUS Intake Valve Cleaner	Medium Petroleum Distillate
004	Shellsol D43	Medium Petroleum Distillate
064	Whitaker Paint Thinner/Mineral Spirits	Medium Petroleum Distillate
091	E-Z Paint Thinner	Medium Petroleum Distillate
131	Gum Turpentine	Miscellaneous
016	STP Octane Booster	Miscellaneous
182	Prestone Heavy Duty Brake & Parts Cleaner	Miscellaneous
039	Pennzoil Roadside Rescue Emergency Fuel Additive	Miscellaneous (Isopar+Aromatic)
042	Chevron Aviation Gasoline 100 LL	Miscellaneous (Isopar+toluene)
146	Sunnyside Brush Cleaner	Miscellaneous (MPD+Aromatic)
010	Cypar 9	Miscellaneous (MPD+Aromatic)
053	Multipurpose Insect Killer	Naphthenic Paraffinic
140	Lamplight Farms Citronella Torch Fuel	Naphthenic Paraffinic
185	Pennzoil Marine Fuel System Cleaner	Naphthenic Paraffinic
201	Summer Lights Citronella Outdoor Lamp Oil	Naphthenic Paraffinic
077	Norpar 12	Normal Alkane
176	V & O Lanterns Candle and Lamp Oil	Normal Alkane
192	Northern Lights Lamp Fuel	Normal Alkane
236	Aura Lamp Oil	Normal Alkane
220	PPG DT 895 Reducer	Oxygenated
069	Whitaker #51 Lacquer Thinner	Oxygenated
218	PPG DT870 Reducer	Oxygenated
231	E-Z Water Wash Brush Cleaner	Oxygenated
149	Sunnyside Denatured Alcohol Solvent	Oxygenated

3.2.2 Methods

3.2.2.1 Weathering Experiments

All weathering experiments were conducted in Dr. Sigman's laboratory at UCF. The ignitable liquids were evaporated (weathered) by volume to approximately 25%, 50%, 75%, 90% and 95% evaporated. Weathering was done in 10 mL vials with 0.1 mL scale divisions. The vial was filled to the 10 mL mark and evaporated to volume while gently heating the sample under a continuous stream of nitrogen to assist in evaporation. The temperature and time conditions for which these ignitable liquids were evaporated were varied based on the overall volatility of the ignitable liquid in question. In each case, the extent of evaporation was monitored by volume measurement of the liquid sample over time. After evaporation was complete, the sample was cooled to room temperature before removing the analytical sample in all cases. At the evaporation volumes of 25%, 50% and 75%, a volume of 20 μL (0.02 mL) was removed by a positive displacement pipette and added to 1 mL of CS_2 for GC-MS analysis. At evaporation volumes of 90% and 95%, a volume of 10 μL (0.01mL) was removed by a positive displacement pipette and added to 0.5 mL of CS_2 for GC-MS analysis. To prepare 0% weathered samples, 20 μL of the ignitable liquid was diluted in 1mL CS_2.

3.2.2.2 Microbial Degradation Experiments

All microbial degradation experiments were conducted at IUPUI. 20μL of the ignitable liquid was spiked onto approximately 100g of potting soil inside a quart-sized paint can. The can was then either immediately extracted as described below or sealed. After storage at room temperature for 7, 14, and 21 days, the can was then re-opened and

a whole carbon strip was suspended on a pre-baked paper clip into the headspace of the can. The can was then resealed and baked at 65°C for 16 hours. Upon cooling the can to room temperature, the carbon strip was removed and cut in half. One half of the strip was stored in an airtight vial and the other half was extracted with 600µL of pentane (or carbon disulfide in the case of oxygenated liquids) and vortexed for 1 minute. A soil control and can control were also prepared. All samples were prepared in duplicate, where one half of the charcoal strip was analyzed at IUPUI and the other was half was sent to UCF for analysis on the same instrument used in the weathering studies. A standard solution of each ignitable liquid was also prepared (0.1% v/v in pentane) for retention time comparisons.

3.2.3 Instrumental Analysis

3.2.3.1 Weathering Experiments

Gas chromatography-mass spectrometry analysis was performed at UCF using an Agilent 6890 gas chromatograph and 5973 mass spectrometer utilizing an auto-sampler. One microliter of the diluted ignitable liquid was injected into a 250°C injection port. The compounds were separated by a 100 % dimethylpolysiloxane (HP-1) capillary column with a film thickness of 0.50µm, a nominal diameter of 200 µm, and 25 m length. Helium gas was maintained at a constant flow rate of 0.8 ml/min with an average velocity of 36 cm/sec. The injection was split in a 50:1 ratio. The initial oven temperature of 50°C was held for 3 minutes, followed by a temperature ramp of 10°C/min to a final temperature of 280°C, which was held for 4 minutes. The mass spectrometer transfer line temperature was 250°C with a source temperature of 230°C and a quadrupole temperature of 150°C.

Mass spectra were acquired between 30 and 350 mass to charge ratio at an acquisition rate of 2-3 scans/second. The detector was tuned off between 1.60 and 2.00 minutes during solvent elution.

3.2.3.2 Microbial Degradation Experiments

All data was acquired at IUPUI using an Agilent 6890 Gas Chromatograph with an Agilent 5975 Mass Spectrometer. The GC was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 micron film thickness). The carrier gas was helium with a flow rate of 1mL/min. The method utilized an inlet temperature of 250°C, 1 µL injection volume, and a 20:1 split ratio. The default oven temperature program started at 40°C for 2 minutes, ramped to 280°C at 10°C/min and held for 3 minutes. The MS parameters included a 3 minute solvent delay and a scan range of m/z 40-300. For oxygenated liquids, a solvent delay was not used. Instead, a timed event was used to turn the detector off at 1.50 minutes and back on at 1.70 minutes. Additionally, the scan range was 24-300m/z initially and then 33-400m/z after the detector was turned back on at 1.70 minutes.

Note that the instrumental parameters for the degraded samples vary slightly from those for the weathered ignitable liquids and therefore result in slight differences in retention time for the same compounds. However, all compounds were identified by mass spectral library search as well as comparison to authentic standards. In addition, the discussion that follows concerns the changes to the chromatographic profiles within a given data set (i.e., either weathering or degradation) not a comparison between laboratories.

3.3 Results and Discussion

The effects of weathering and microbial degradation are two separate processes which can be observed in the chromatograms of the ignitable liquids presented here. In this work, several ignitable liquids in each ASTM class were weathered up to 95% (v/v) via evaporation under nitrogen. In addition, un-evaporated samples were subjected to microbial degradation on potting soil for up to 21 days. The results for one liquid from each ASTM class will be presented and discussed here. The chromatograms for all other liquids listed in Table 3-1 can be found in Appendix A.

Gasoline is the most common ignitable liquid used in incendiary fires. Gasoline (Figure 3-1) contains a significant contribution of aromatic compounds but also contains normal, branched and cyclic alkanes with a minor contribution of naphthalenes. The set of chromatograms on the left shows the effects of weathering while the set of chromatograms on the right shows the effects of microbial degradation. As demonstrated in Figure 3-1, in weathering, gasoline suffers from the loss of the branched alkanes that appear early in the chromatogram in addition to toluene and the C_2-alkylbenzenes. Weathering also results in an increasing relative abundance of heavier components such as 1,2,4-trimethylbenzene and naphthalene compounds. In contrast, in microbial degradation, gasoline suffers from the loss of selected compounds based on chemical structure, not boiling point. The compounds that are degraded first include the normal alkanes, toluene, ethylbenzene and propylbenzene. The ratios of the C_2- and C_3-alkylbenzenes are also significantly altered after 7 days. The C_3-alkylbenzenes must be present in a fire debris sample in order to identify an ignitable liquid residue as being gasoline, the ratio of these compounds must be consistent with that of a gasoline standard.

The change in ratios of the C_3-alkylbenzenes therefore interferes with the identification of gasoline after a few days on soil.

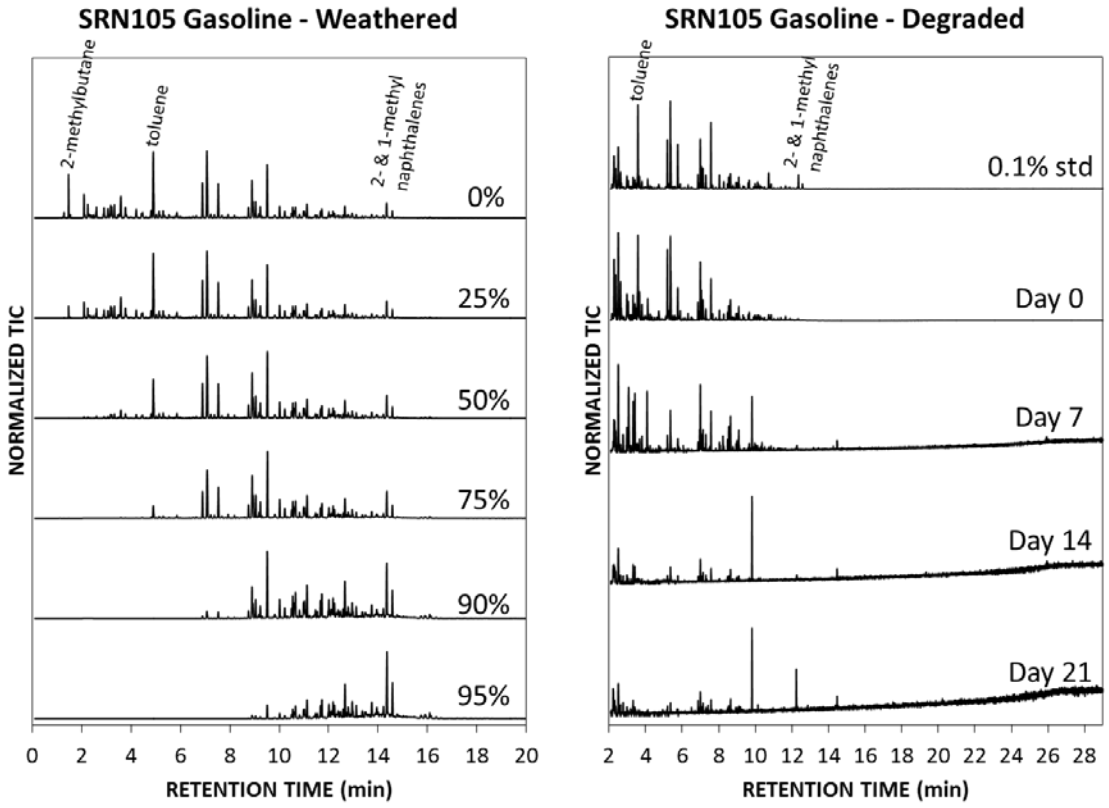


Figure 3-1 The effects of weathering versus microbial degradation of gasoline.

Figure 3-2 demonstrates the effects of weathering compared to microbial degradation of a heavy petroleum distillate (HPD). Weathering of an HPD results in the loss of all compounds including aromatics and all alkanes in the leading edge of the chromatogram. As the severity of the weathering increases, more and more of the less volatile compounds are lost increasing the relative intensity of the less volatile compounds left behind. The result of this phenomenon is a shift of the distribution of the n-alkanes compared to a pristine heavy petroleum distillate. However, in an HPD sample

that has been exposed to bacteria, the n-alkanes are quickly degraded, resulting in an unresolved alkane envelope comprised predominantly of branched and cyclic alkanes. It is interesting to see, however, that there appears to be a systematic shift towards compounds with higher boiling points in degraded samples. This phenomenon should be further investigated to determine if this phenomenon was an artifact of simultaneous weathering. This would be possible if all cans were not completely sealed. This phenomenon could also occur if the bacteria have a preference for the lower boiling compounds. It could also be a soil adsorption effect; however, it would be more likely if the shift were toward the lower boiling compounds as heat would drive these compounds out of the soil better than the heavier compounds. It is also worth noting that the safrole peak that dominates the chromatogram at 21 days is believed to be from the presence of wood fragments found in the potting soil.

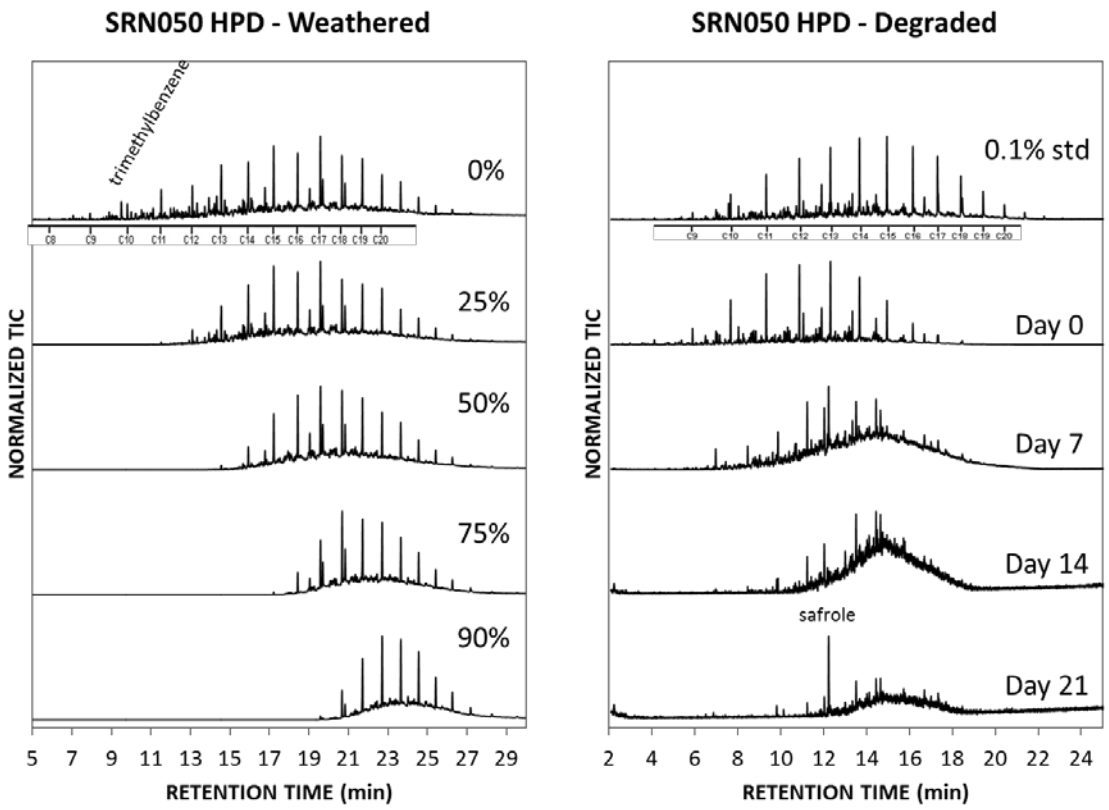


Figure 3-2 The effects of weathering versus microbial degradation of a heavy petroleum distillate.

The effects of weathering and microbial degradation of an aromatic liquid are shown in Figure 3-3. This aromatic liquid is comprised predominantly of alkyl benzenes with smaller contributions of naphthalene compounds. As the aromatic liquid is weathered, the trimethylbenzenes, which are the lightest components in the sample, are the first to be affected followed by the lower boiling alkylbenzenes. As these components are lost, a relative increase in abundance of the higher boiling alkylbenzenes and naphthalenes is observed. This trend is not observed in the degradation of the aromatic liquid. Rather, while the trimethylbenzenes are lost, the C_4-alkylbenzenes remain with some changes in the ratios among these compounds. The changes in the ratios of these

components suggest that the earlier eluting C_4-alkylbenzenes, which were affected by weathering, suffer less from microbial degradation than the later eluting C_4-alkylbenzenes. Additionally, while an increase in abundance of the napthalenes and pentamethylbenzene is observed in the highly weathered samples, the relative abundance of these compounds remains fairly consistent in the degraded samples.

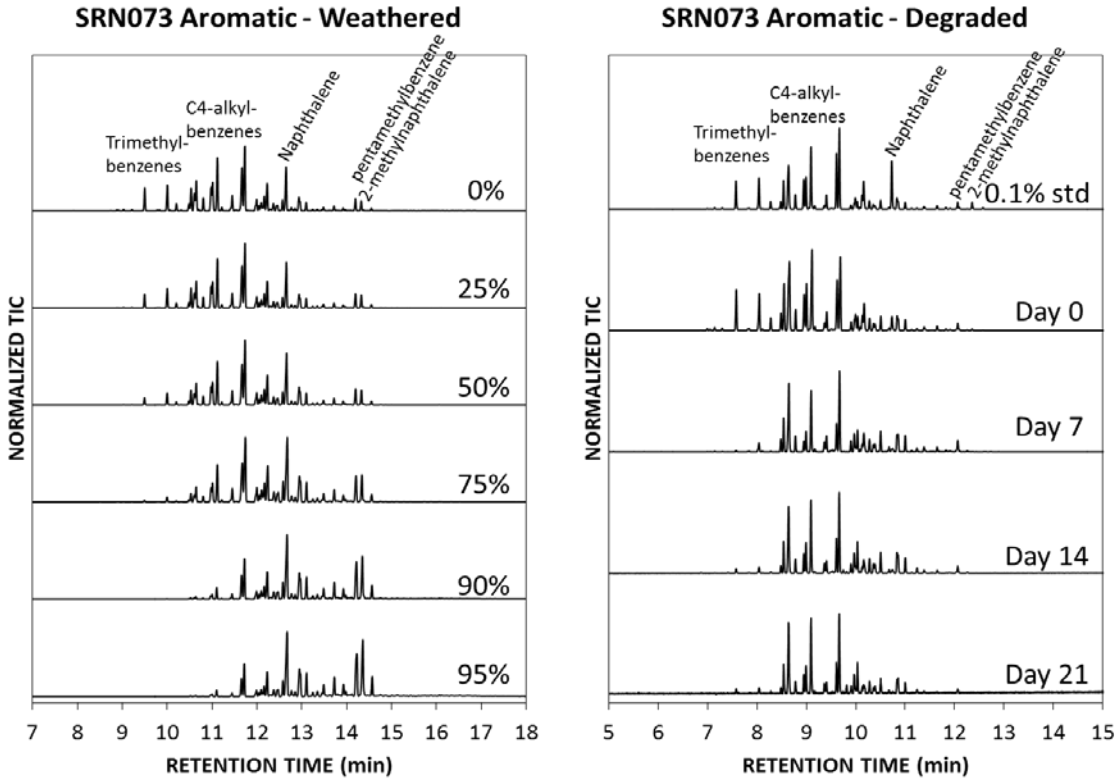


Figure 3-3 The effects of weathering versus microbial degradation of an aromatic product.

Figure 3-4 demonstrates the effects of weathering and microbial degradation on a miscellaneous liquid which contains a mixture of an aromatic and a heavy petroleum distillate. At 25% weathered, the C_2-alkylbenzenes are lost first, with some loss of the C_3-alkylbenzenes and a slight increase in relative abundance of the alkane portion. Total loss of the C_3-alkylbenzenes is observed at 50% weathered while the alkane portion

remains largely unweathered. However, at 75% weathered a significant decrease in n-C_{11} and n-C_{12} is observed and at 90% weathered, a clear shift in the chromatographic profile of the n-alkanes is observed. A quite different trend is noted in the microbial degradation of this miscellaneous liquid. After exposure to the soil for 7 days, the C_2-alkylbenzenes are completely lost and the C_3-alkylbenzenes are significantly reduced. In addition, the n-alkanes also suffer from bacterial action. After 14 days, the bacteria have almost completely metabolized the C_3-alkylbenzenes and all the n-alkanes. Finally after 21 days, all that is left is an unresolved alkane envelope and dimethyl disulfide, a compound produced by the bacteria in the soil.

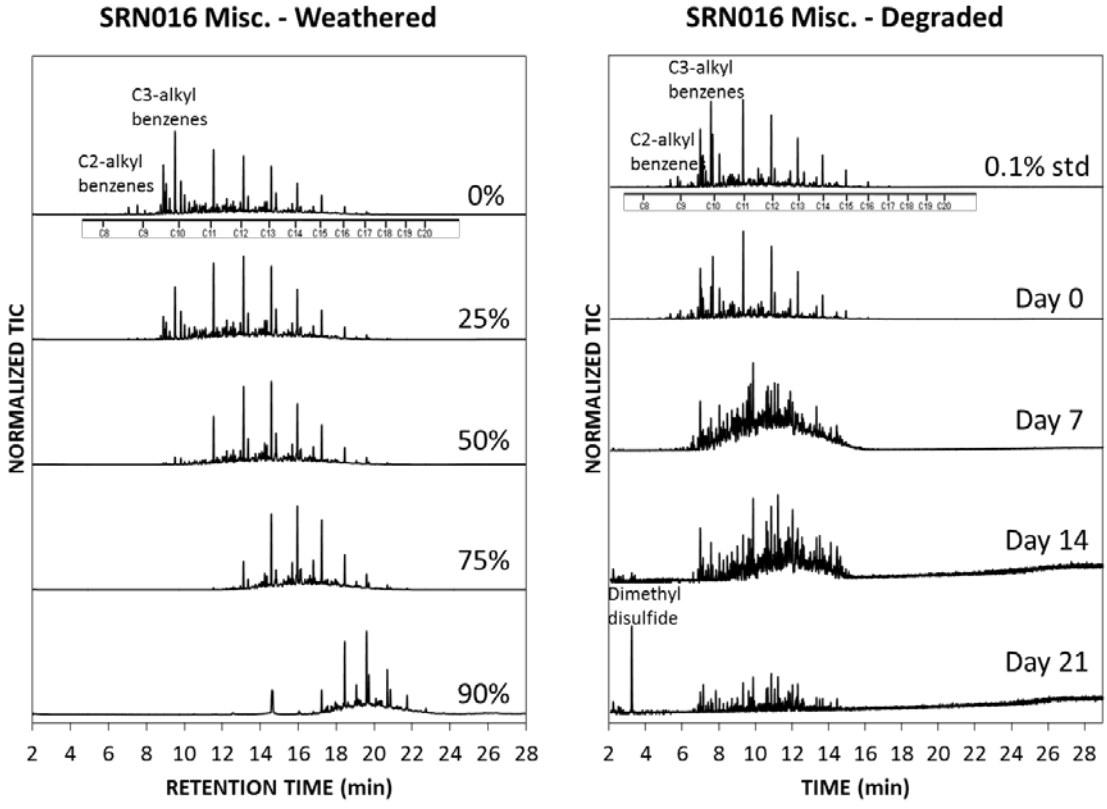


Figure 3-4 The effects of weathering versus microbial degradation of a miscellaneous liquid.

The effects of weathering and microbial degradation of an isoparaffinic product are shown in Figure 3-5. As weathering increases, a loss of the lower boing compounds are observed, while the relative abundance of the lesser volatile compounds increases. For example, the trimethylpentanes that are most abundant in the 0% weathered sample become less abundant while the trimethylhexanes, trimethylheptanes, and trimethyloctanes increase in abundance. However, in microbial degradation of this isoparaffinic product, the trimethylpentanes remain largely unaffected, while the trimethylhexanes, trimethylheptanes, and trimethyloctanes are almost completely degraded over the course of 21 days. While bacteria preferentially degrade n-alkanes and aromatics, when presented with a liquid comprised of only branched alkanes, they will degrade the long chain, lesser branched alkanes such as the trimethyloctanes found in the isoparaffinic liquid shown in Figure 3-5.

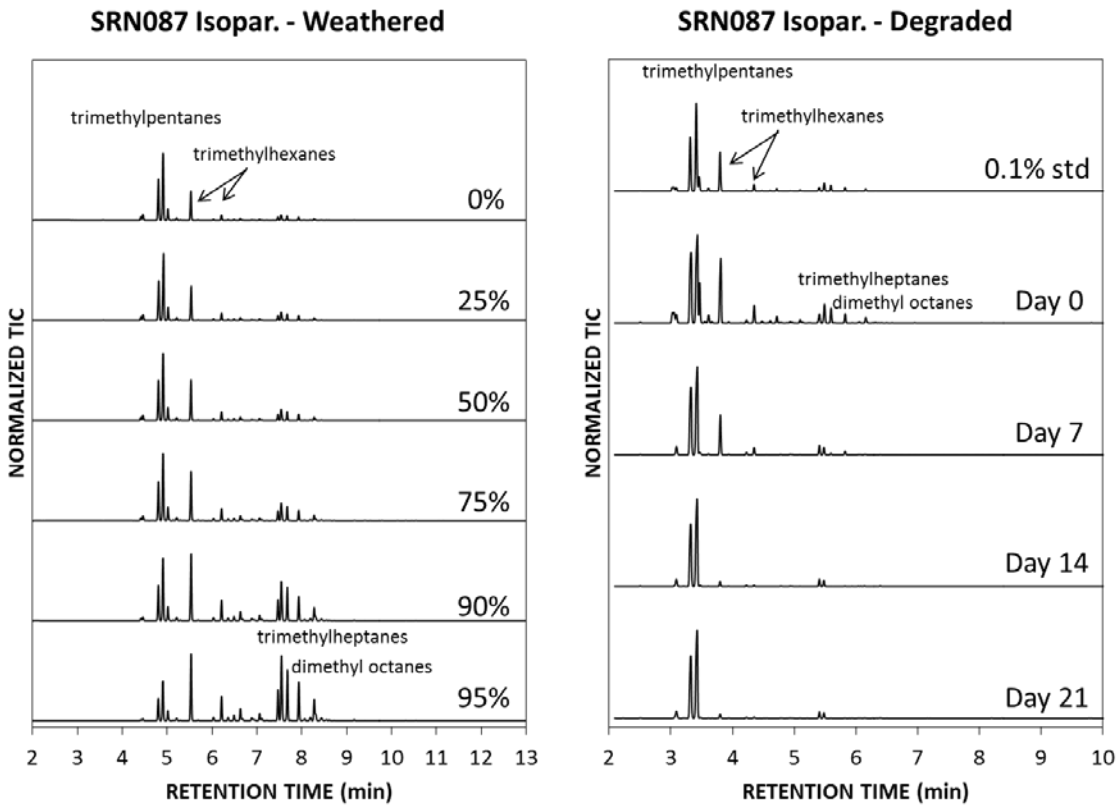


Figure 3-5 The effects of weathering versus microbial degradation of an isoparaffinic product.

Figure 3-6 demonstrates the effects of weathering and microbial degradation of a normal alkane product. In weathering, the more volatile n-alkanes are reduced while the less volatile ones increase in relative abundance. In microbial degradation, a significant loss of the lesser abundant alkanes (n-C_{10} and n-C_{13}) are observed while the more abundant alkanes remain largely unaffected. However, upon careful observation of the chromatogram at day 7 compared to day 0, it is clear that even the significantly abundant alkanes are being reduced in abundance. In day 0, n-C_{11} and n-C_{12} have broad peak widths, however, in day 7 the peak widths are much narrower, suggesting the abundance of these compounds is significantly lower than they were in day 0.

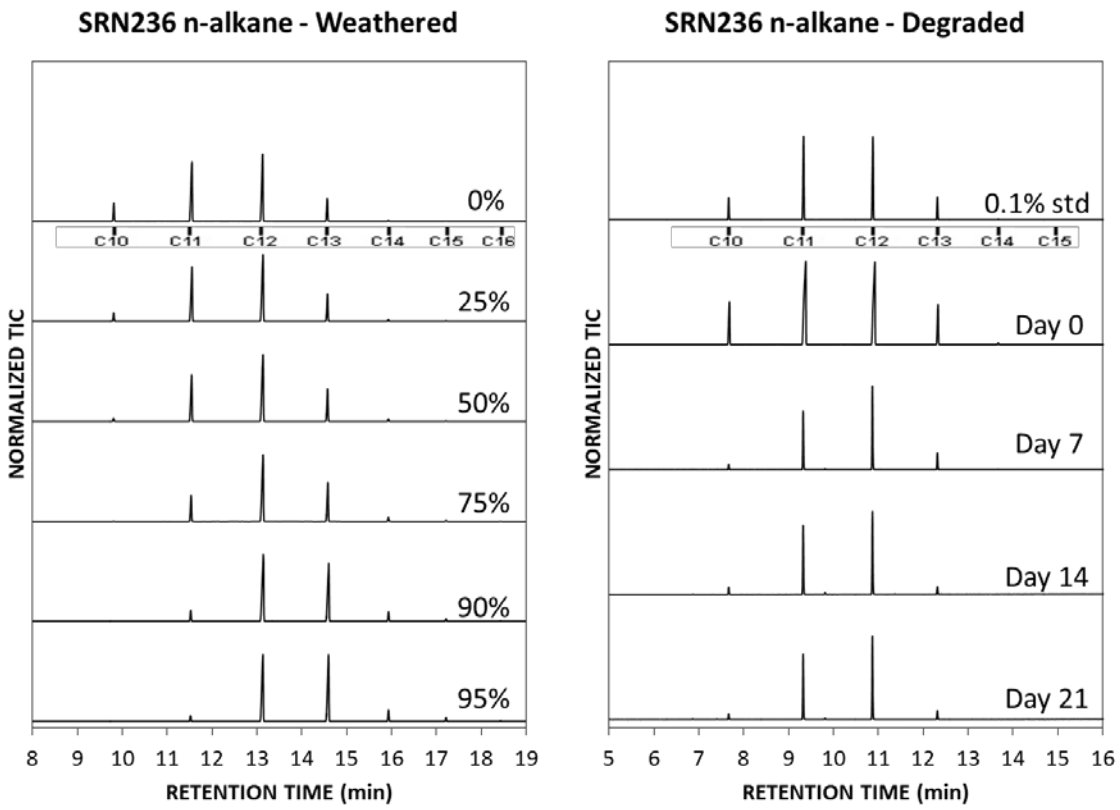


Figure 3-6 The effects of weathering versus microbial degradation of a normal alkane product.

The naphthenic-paraffinic product shown in Figure 3-7 is not as significantly affected by weathering and microbial degradation. The naphthenic-paraffinic product is comprised largely of branched alkanes and naphthenic compounds such as 2-methyl-trans-decalin. This liquid does contain a small contribution of n-alkanes. In weathering, the n-C_{11} and 2-methyl-trans-decalin as well as the earlier eluting compounds are lost due to evaporation while the less volatile compounds, including n-C_{12}, show an increase in relative abundance. On the other hand, both n-C_{11} and n-C_{12} are the first to be degraded by the bacteria, while the relative abundance of 2-methyl-trans-decalin increases.

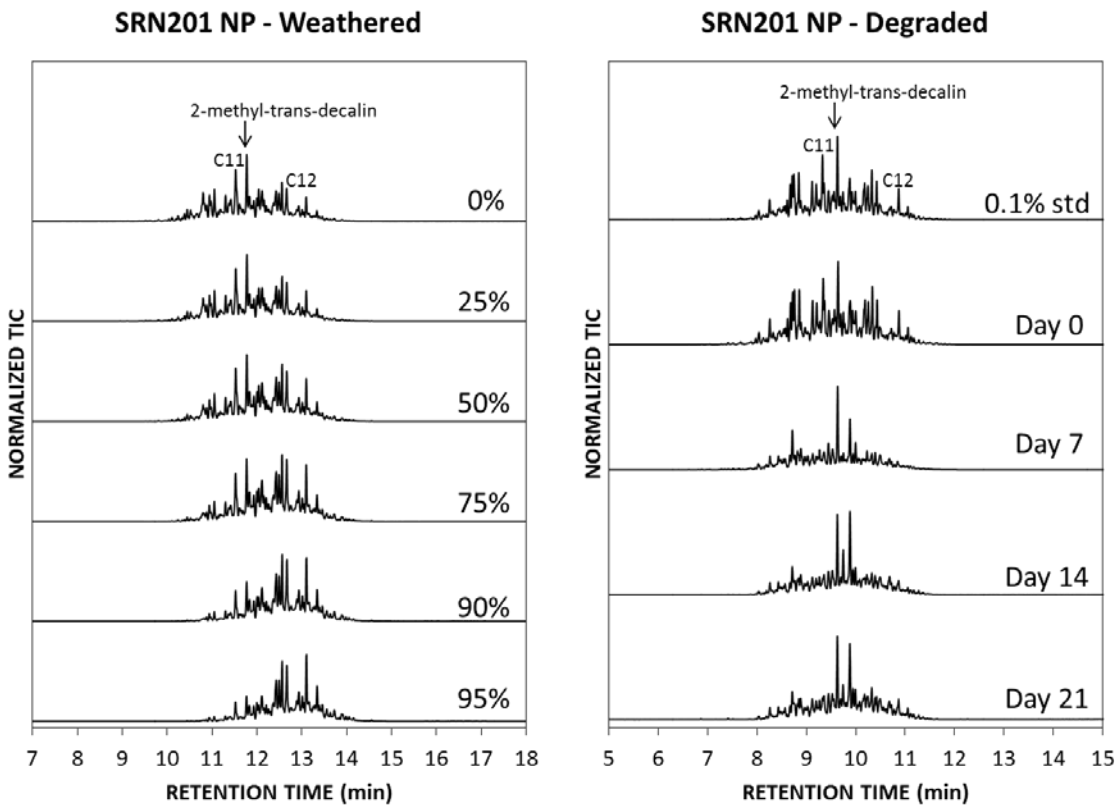


Figure 3-7 The effects of weathering versus microbial degradation of a naphthenic-paraffinic product.

Figure 3-8 demonstrates the effects of weathering and microbial degradation of an oxygenated liquid. This liquid contains 2 oxygenated species, isopropanol (IPA) and butanone, which are the first two compounds to elute. This liquid also contains aromatic and aliphatic compounds. When this liquid was subjected to weathering, a loss of compounds based on boiling point was observed, where IPA and butanone decreased quickly followed by heptane and methylcylcohexane. At 95% weathered, toluene and very small amounts of heptane and methylcylcohexane remained. However, in microbial degradation, all compounds are lost after 7 days. The only compound visible in the chromatogram is pentane, which is believed to be residual solvent from the syringe wash,

not a lack of bacterial action on this compound. The oxygenated compounds as a whole were difficult to recover from soil samples. Experiments were repeated with an adjusted passive headspace method consisting of 85°C for 4 hours. The data from these experiments is shown in Appendix B.

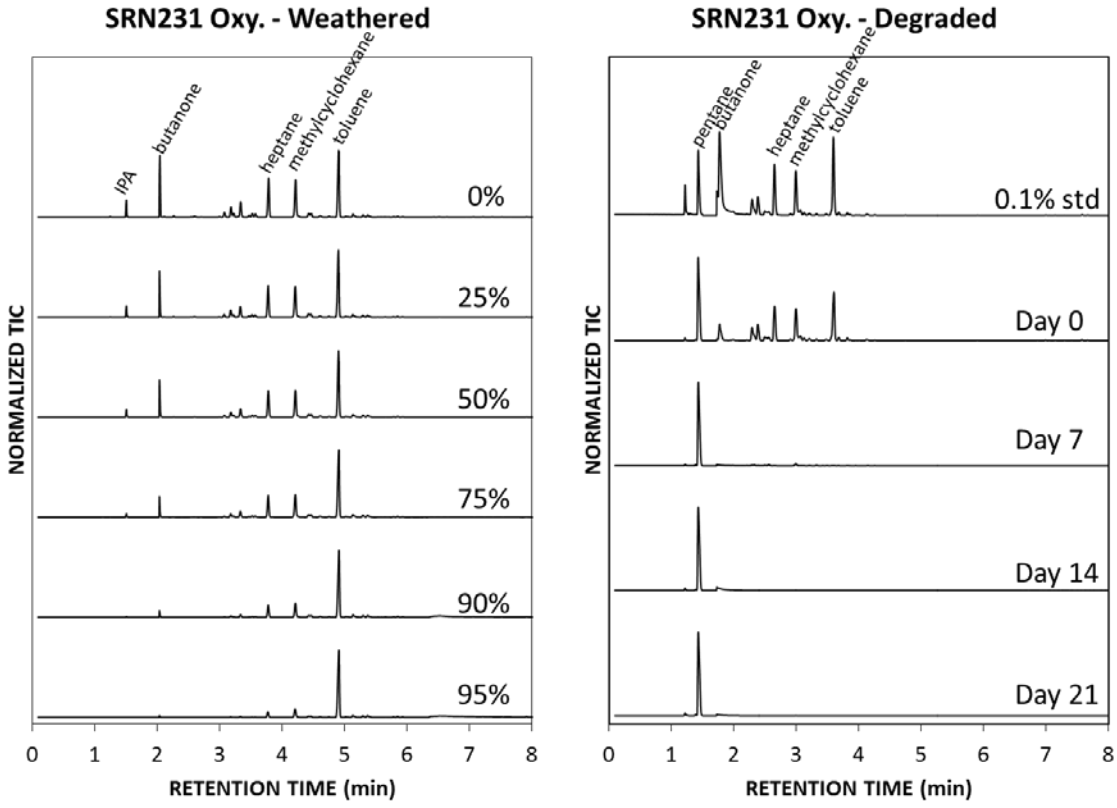


Figure 3-8 The effects of weathering versus microbial degradation of an oxygenated product.

3.4 Conclusions

Microbial degradation is based on the ability of bacteria to metabolize the compounds in ignitable liquids, whereas weathering results in the loss of all lower boiling compounds with no particular preference based on chemical structure. Bacteria prefer to utilize n-alkanes and lesser substituted alkylbenzenes. Among the alkybenzenes, toluene is degraded first, followed by the C_2-alkylbenzenes, then the C_3-alkylbenzenes, and

finally the C_4-alkylbenzenes. Long chained, lesser branched alkanes are also susceptible to microbial attack, although in a sample containing alkylbenzenes and n-alkanes, these branched alkanes are not preferred. All ignitable liquids examined suffered at least to some extent from microbial degradation, although gasoline, petroleum distillates, and oxygenates suffered the most, while the isoparaffinic and naphthenic paraffinic products were effected the least.

CHAPTER 4. CHARACTERIZING MICROBIAL DEGRADATION

4.1 Introduction

Identifying ignitable liquid residues (ILR) such as gasoline in fire debris samples using gas chromatography-mass spectrometry (GC-MS) is an important part of an arson investigation. Currently, one of the most popular methods for isolating ILR from fire debris is passive adsorption onto an activated charcoal strip, followed by solvent elution of the ILR using carbon disulfide, pentane or another appropriate solvent. This technique has been in use for many years and its performance has been well studied [40-43].

Following isolation of the ILR, analysis by GC-MS is used almost universally to detect and classify the type of ignitable liquid that may be present. Extracted ion profiles that are characteristic of particular compound classes are generated (i.e., alkanes, aromatics, cycloparaffins, indanes, and polynuclear aromatics) [41, 44, 45]. In addition, specific target compounds are identified by their mass spectra and retention times. In particular, 2-ethyltoluene, 3-ethyltoluene, 4-ethyltoluene, 1,3,5-trimethylbenzene and 1,2,4-trimethylbenzene must be present according to the ASTM standard governing the analysis of ILR by GC-MS [1].

There are several challenges to the interpretation of GC-MS results. One of the most common is the presence of a large background of pyrolysis products that can obscure ILR patterns [46, 47]. Extracted ion profiles are specifically intended to filter out

these signals. Another challenge is weathering, which distorts the ILR due to evaporation or partial burning and results in loss of low boiling compounds. Comparison of a weathered sample to a library of weathered exemplars can assist in the interpretation of these results. Chemical markers of weathered gasoline have also been reported [48].

Less commonly, microbial degradation can occur in samples rich in organic matter such as soil. Microbial degradation of compounds found in petroleum is a well known phenomenon in environmental science where indigenous bacteria can metabolize petroleum compounds in order to remediate contaminated areas [49-53]. In contrast, microbial degradation of hydrocarbons in fire debris is problematic. In these cases, the identification of ignitable liquid residues can become difficult or even impossible given enough microbial action. The forensic literature contains several examples where microbial degradation of ignitable liquids has been studied in controlled laboratory experiments [12, 30, 32, 33]. Bacteria from the genus *Pseudomonas*, which are believed to be largely responsible for biodegradation in fire debris, have been cultured and identified by Kirkbride [12].

The complex nature of gasoline and other ignitable liquids makes statistical approaches attractive for the data analysis of fire debris samples. A recent review by Sandercock discusses the many statistical approaches that have been applied to the analysis of various neat and weathered ignitable liquids [15] and one of the most common statistical methods being Principal Component Analysis (PCA) [16-19]. PCA is a multivariate statistical technique that simplifies a complex data set into fewer dimensions that can be used to visualize trends in the data. Overall, these studies have been focused

on the chemical fingerprinting of ignitable liquids, with a particular emphasis on discriminating and identifying different ignitable liquids in varying stages of weathering.

To date, the microbial degradation of gasoline and other ignitable liquids has been well-studied under laboratory conditions [12, 14, 30, 32, 54, 55]. However, microbial degradation has not been studied under realistic conditions where the ignitable liquid has also been subject to weathering from a fire. In addition, the nature and extent of microbial degradation could change based on populations of bacteria in the soil, which may be dependent on soil type and season. The type of sample that is collected from an incendiary device, such as a Molotov cocktail (i.e., glass fragments versus cloth wick versus soil) may play a large role in the rate in which microbial degradation of the ignitable liquid occurs. Therefore, this study was designed to detect any differences in the microbial degradation of gasoline from Molotov Cocktails using differing soil types, substrates, amounts of gasoline, and during different seasons. Lastly, this work utilizes multivariate statistics to illustrate these differences and determine what chemical changes are most significant.

4.2 Materials and Methods

4.2.1 Chemicals

Gasoline (87 octane, unleaded) was obtained locally. Pentane was obtained from Fisher Scientific. Standards of various alkane and aromatic compounds identified in gasoline were purchased from VWR, Sigma-Aldrich, and Fisher. Activated charcoal strips and unlined quart-sized paint cans were obtained from Albrayco Technologies and Lab Safety Supply, respectively. Hyponex® brand potting soil and was obtained from Wal-Mart.

4.2.2 Weathering Study

Approximately 2 mL of fresh gasoline was transferred to each of 15 culture tubes. The tubes were then capped and the mass of the gasoline in each tube was determined. The caps were then removed from twelve of the tubes, which were placed under a stream of nitrogen until approximately 25, 50, 75, and 90% of the original volume was evaporated (three replicates per level). The evaporated samples were then re-capped and weighed again to obtain the mass of evaporated gasoline. Unweathered samples were allowed to stand during the evaporation of the other samples with the caps remaining tightly in place. 20 μL of each sample was spiked onto a Kimwipe in a quart-sized paint can and analyzed by passive headspace adsorption followed by solvent elution.

4.2.3 Laboratory Study

20 μL aliquots of gasoline were spiked onto approximately 90 grams of potting soil and stored in a sealed quart-sized paint cans for 0, 7, 11 and 22 days. The samples, as well as a soil control and a can control, were then analyzed using passive headspace adsorption followed by solvent elution. Briefly, one third of a charcoal strip (~7 × 9 mm^2) was suspended on a pre-baked paper clip using a strand of nylon string. The can was sealed and baked in an oven at 85°C for 4 h. After cooling the can to room temperature, the carbon strip was removed. The gasoline was eluted off the strip by adding 400 μL of pentane to the strip in a small test tube and vortexing for approximately 1 minute. The samples were then subjected to GC/MS analysis.

4.2.4 Field Studies

Two separate studies were conducted, one in July and one in January. For each study, four Molotov Cocktails were assembled and deployed by the Indianapolis Fire

Department (IFD), using 2 wine bottles and 2 beer bottles filled to the neck with gasoline. Two of these Molotov Cocktails (1 of each bottle size) were deployed on areas covered with approximately 6-8 lbs of potting soil spread out around a concrete block. The other two were deployed onto each of two areas of native lawn soil that were approximately 2'W x 2'L, where the first 3 inches of grass and soil was removed. Soil samples were collected prior to the burn to serve as controls. Each Molotov cocktail was allowed to burn until it self-extinguished. The glass fragments at each site were first collected in gallon paint cans. Then approximately 2 gallons of soil at each site was collected into 5 gallon steel pails and manually mixed in an effort to homogenize the sample. Then, the soil was separated into 24 quart cans, filling each can with 1-2 inches of soil.

All samples were stored at room temperature until analysis after 0, 2, 7, 11, 22, 29, 45, and 60 days, with the exception of the winter study, where the sixth time point was 32 days instead of 29 days. All soil samples were analyzed in triplicate. The ignitable liquid residues were extracted from the soil using passive headspace adsorption onto a charcoal strip. At each time point, samples of the soil control and glass fragments were removed from their respective gallon cans and placed into clean quart cans and subjected to passive headspace analysis. The original cans for the glass fragments and soil controls were then resealed and stored until the next time point. After heating at 85°C for 4 h, the samples were extracted with 400 μL of pentane. A standard solution of gasoline (0.1% v/v in pentane) as well as a standard hydrocarbon mixture (0.01% v/v in pentane) containing only the analytes of interest was also prepared for retention time comparisons. All samples were then subjected to GC/MS analysis.

4.2.5 GC/MS Method

All data was acquired using an Agilent 6890 Gas Chromatograph with an Agilent 5975 Mass Spectrometer. A Gerstel MPS2 autosampler was used for liquid injections. The GC was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 µm). The carrier gas was helium with a flow rate of 1 mL/min. The method utilized an inlet temperature of 250 °C, 1 µL injection volume, and a 20:1 split ratio. The default oven temperature program started at 40 °C for 3 min, ramped to 280 °C at 10 °C/min, and held at 280 ^{o}C for 3 min. The MS parameters included an appropriate solvent delay and a scan range of m/z 40-300.

4.2.6 Data Analysis

Each component was identified based on comparison of its retention time and mass spectrum to authentic standards and the National Institute of Standards and Technology mass spectral database. Summed Extracted Ion Profiles (EIPs) were generated corresponding to characteristic fragments of either n-alkanes (m/z 57, 71, 85, and 99) or aromatics (m/z 91, 105, and 119). The peak areas in each of the EIPs were generated using the Xcaliber data analysis software (Thermo Scientific). Peaks that were not visible in the extracted ion chromatogram were recorded as having no peak area. In practice, extracted ion profiles allow the analyst to filter out signals from pyrolysis products or other contaminants that can interfere with the ability to identify the ignitable liquid.

To pre-treat the data for statistical analysis by Principal Components Analysis (PCA), the peak areas from the EIPs were normalized for unit vector length and then autoscaled. To normalize the data, the peak area for a given compound in a sample was

divided by the square root of the sum of the squares of the peak areas for all samples. This normalized peak area was then autoscaled by subtracting the mean peak area for the compound across all samples and then dividing by the standard deviation of the peak area across all samples. Normalizing and autoscaling eliminate variability in peak area due to sample concentrations and allow the variance of variable to be weighted equally [56-59].

Principal Components Analysis (PCA) was then performed on the weathered and degraded chromatograms separately, followed by the combined data set of both weathered and degraded chromatograms. The software used was XLSTAT (AddinSoft), an add-in for Microsoft Excel. PCA was also performed on all data generated from the soil samples generated from the Molotov Cocktails. PCA generates non-correlated factors that are linear combinations of the original variables. The factor loadings plot shows the correlation between each variable (compounds of interest) and these non-correlated factors. The factor scores (observations) plot shows the total contribution of the variables to each of the samples. PCA was used to show trends in the data that may not be realized by visual inspection due to the sheer number of variables as well as the large number of samples. In this case, the variables were the 20-22 relevant compounds in gasoline that are readily degraded compared to the other hundreds of compounds in gasoline. These compounds of interest include normal alkanes from C_7 to C_{18}, as well as toluene, the C_2-alkylbenzenes and C_3-alkylbenzenes. Using the scree plot from PCA, it was determined that the first three principal components were significant. The software used was XLSTAT (AddinSoft), an add-in for Microsoft Excel. For all PCA plots, the open data points had a negative score along the third principal component and the filled data points

had a positive score along the third principal component, which allows us to show that there is some separation in the third dimension.

4.3 Results and Discussion

4.3.1 Laboratory Study

A comparison of the effects of weathering and microbial degradation on the chromatographic profiles of fresh gasoline is shown in Figure 4-1. Weathering does not discriminate against chemical motifs, such as alkanes or aromatic hydrocarbons; rather it discriminates against boiling point [32]. Therefore, a severely weathered gasoline sample (as shown in Figure 4-1d) will have little to none of the lower boiling compounds, such as toluene (peak 1) and n-octane (peak 2), and an increased relative abundance of the higher boiling compounds, such as 1,2,4-trimethylbenzene (peak 10). However, in microbial degradation, the bacteria selectively utilize the hydrocarbons based on chemical structure, such as n-C_8 (peak 2), ethylbenzene (peak 3), propylbenzene (peak 6), and n-C_{10} (peak 11), as shown in Figure 4-1e-h.

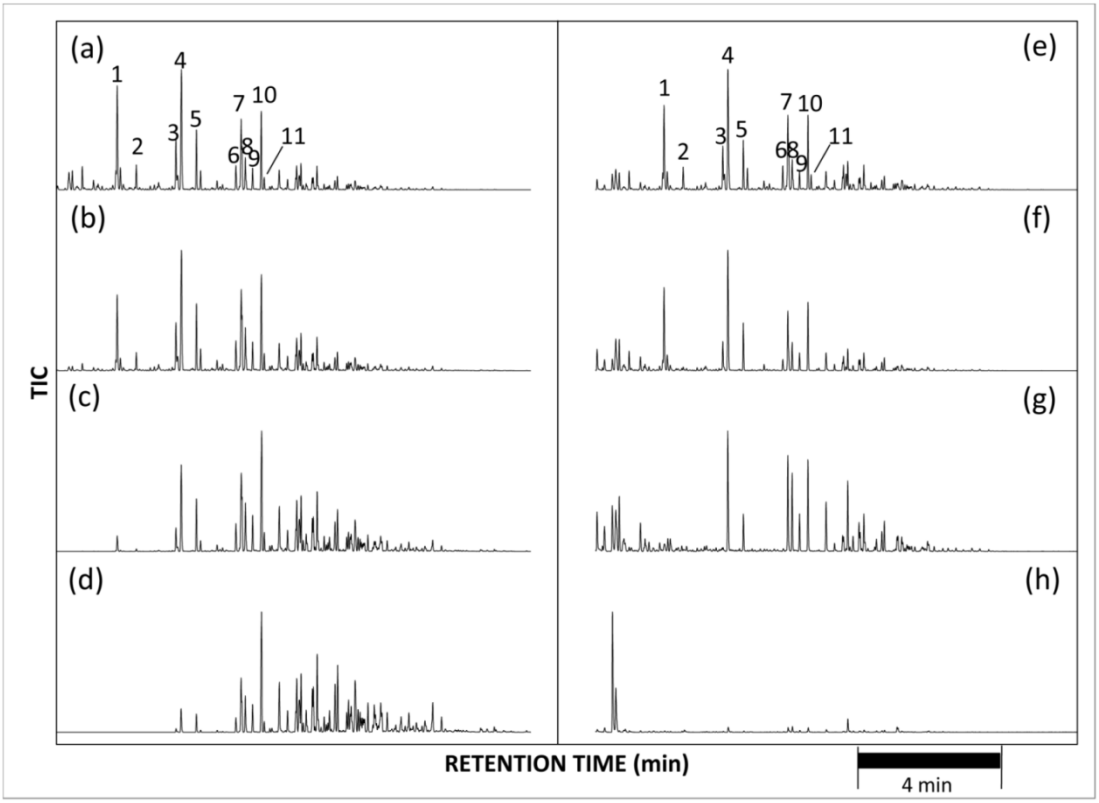


Figure 4-1 TIC of fresh gasoline after weathering (a) 0%, (b) 50%, (c) 75%, (d) 89% and microbial degradation after (e) 0 days, (f) 2 days, (g) 7 days, and (h) 14 days. Peaks: (1) n-C_7, (2) toluene, (3) n-C_8, (4) ethylbenzene, (5) *m*- and *p*-xylene, (6) *o*-xylene, (7) n-C_9, (8) isopropylbenzene, (9) propylbenzene, (10) 3- and 4-ethyltoluene, (11) 1,3,5-trimethylbenzene, (12) 2-ethyltoluene, (13) 1,2,4-trimethylbenzene, and (14) n-C_{10}.

To ensure that ignitable liquids are being degraded instead of adsorbed by the organic matter that makes up soil, gasoline was spiked onto autoclaved (sterilized) soil [32]. This comparison showed that the losses observed in the live soil were due to microbial action, not due to soil adsorption effects.

Following PCA, the observations for weathered samples were projected into the space defined by their first two principal components, as shown in Figure 4-2. In this case, 97.5% of the variance is displayed. Inspection of the observations shows that they are

largely separated along the first principal component, which represents 79.68% of the variance. The factor loadings of the variables (the peak areas for the alkane and aromatic compounds) are shown in Figure 4-3. The factor loadings indicate that those compounds whose boiling points are less than approximately 160 ^{o}C are associated with fresh samples (i.e., those samples that are less than 60% weathered). Compounds whose boiling points are greater than approximately 160 ^{o}C are associated with highly weathered samples.

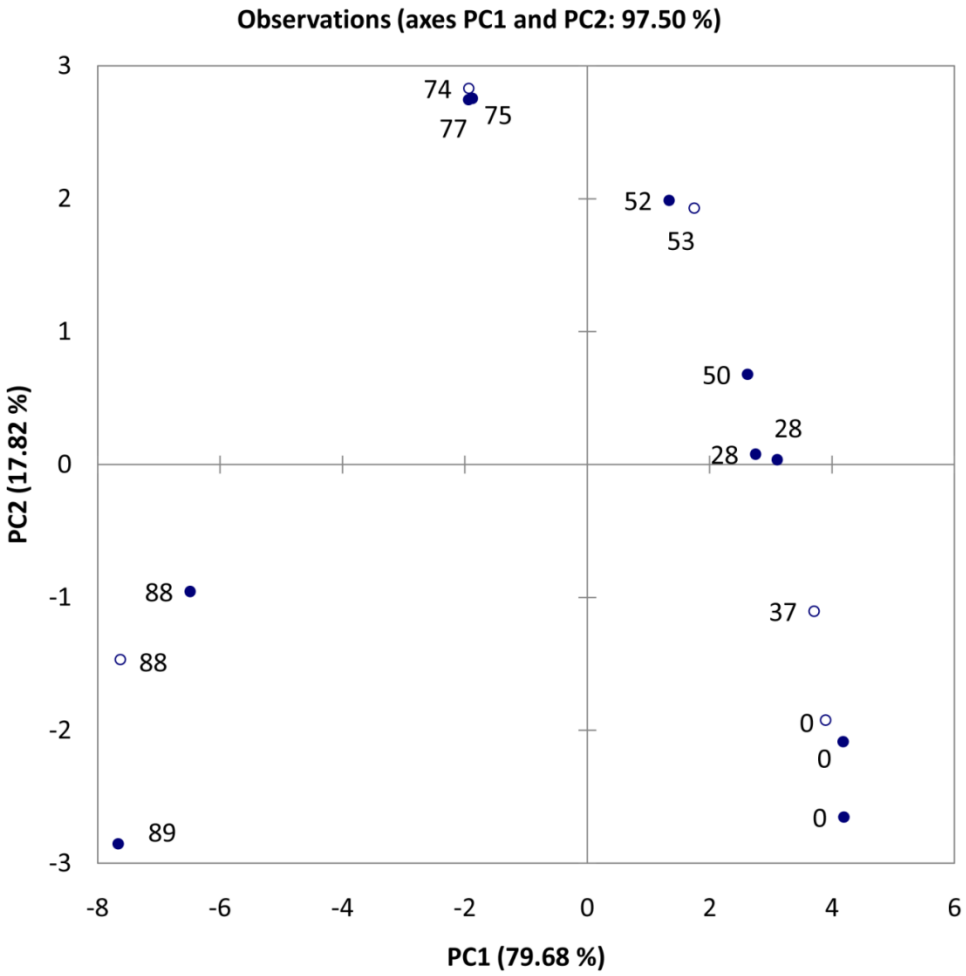


Figure 4-2 Plot of a set of weathered gasoline samples as projected into the first two principal components.

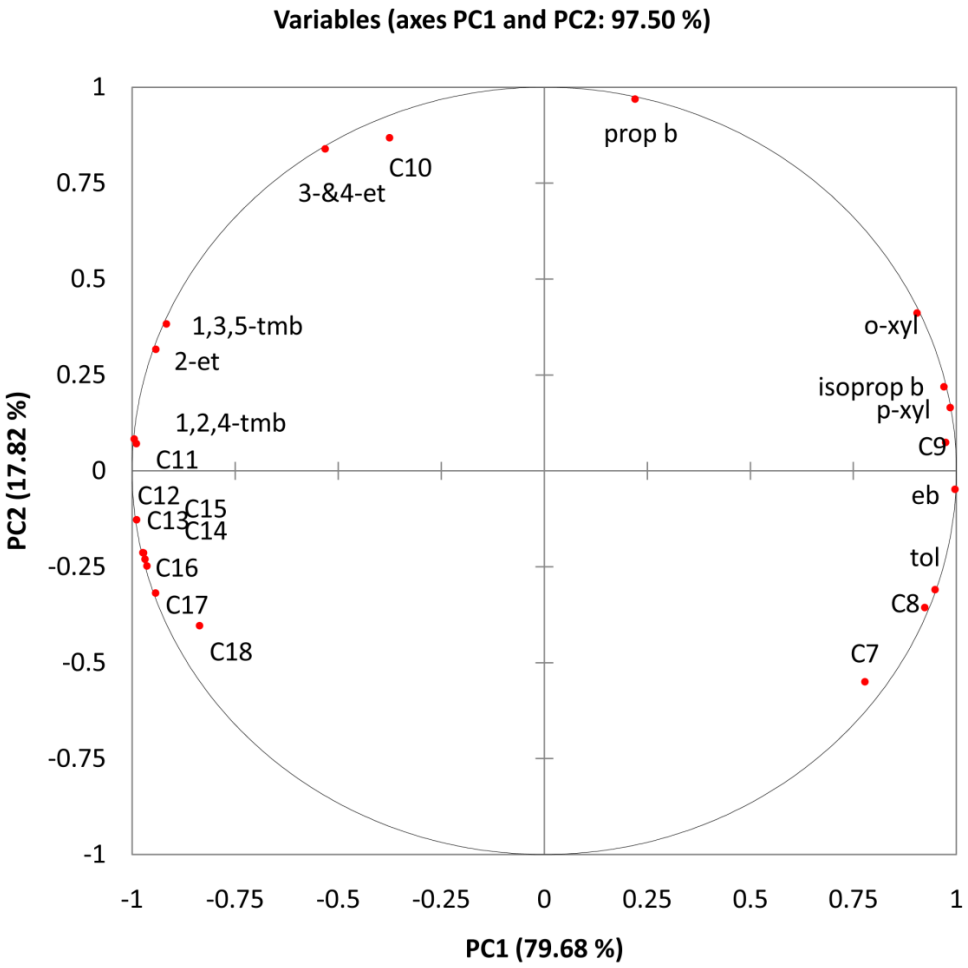


Figure 4-3 Plot of the factor loadings for a set of weathered gasoline samples.

The observations for biodegraded samples are projected into the space defined by the first two principal components in Figure 4-4. In this case, 80.66% of the variance is displayed. Inspection of the observations shows that they are not separated along only one principal component; rather they form an arc across the two-dimensional space. The factor loadings, as shown in Figure 4-5, indicate that the normal alkanes are strongly correlated with one another as well as being associated with fresh samples. This agrees

with the observation that the n-alkanes are among the first compounds to be degraded in a petroleum sample [12, 30, 32, 33]. In contrast, 2-ethyltoluene and 1,3,5-trimethylbenzene are associated with highly degraded samples. This agrees with the observation that highly substituted aromatics are resistant to microbial degradation [12, 30, 32, 33].

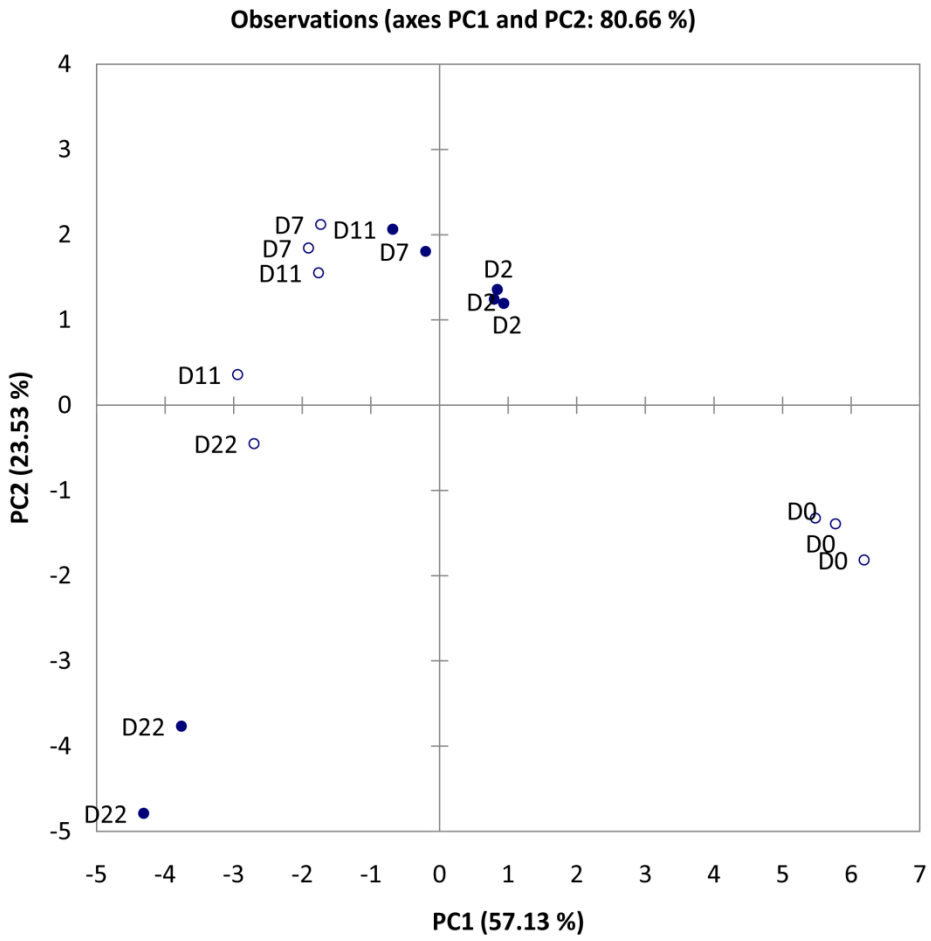


Figure 4-4 Plot of a set of biodegraded gasoline samples as projected into the first two principal components.

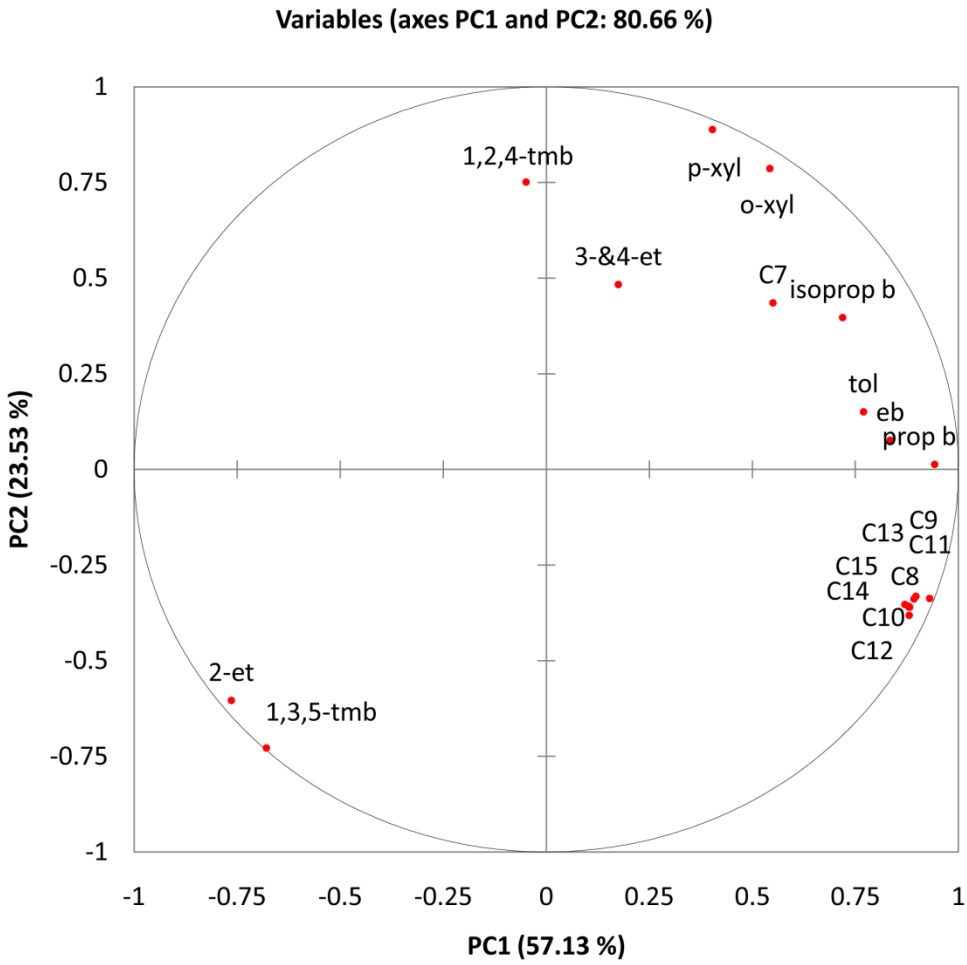


Figure 4-5 Plot of the factor loadings for a set of biodegraded gasoline samples.

Lastly, the observations from both weathered and degraded samples projected into the same space defined by the first two principal components for this data set is shown in Figure 4-6, where 80.31% of the variance is displayed. In this plot, two completely different trends are observed for weathering versus microbial degradation. The factor loadings, as shown in Figure 4-7, result in a group of compounds in the upper left quadrant that have a relatively low boiling point and also are degraded rather quickly.

Compounds in the right two quadrants have higher boiling points and are either not degraded at all (e.g., 1,3,5-trimethylbenzene and 2-ethyltoluene) or are not degraded as quickly (e.g., 3- and 4-ethyltoluene and 1,2,4-trimethylbenzene).

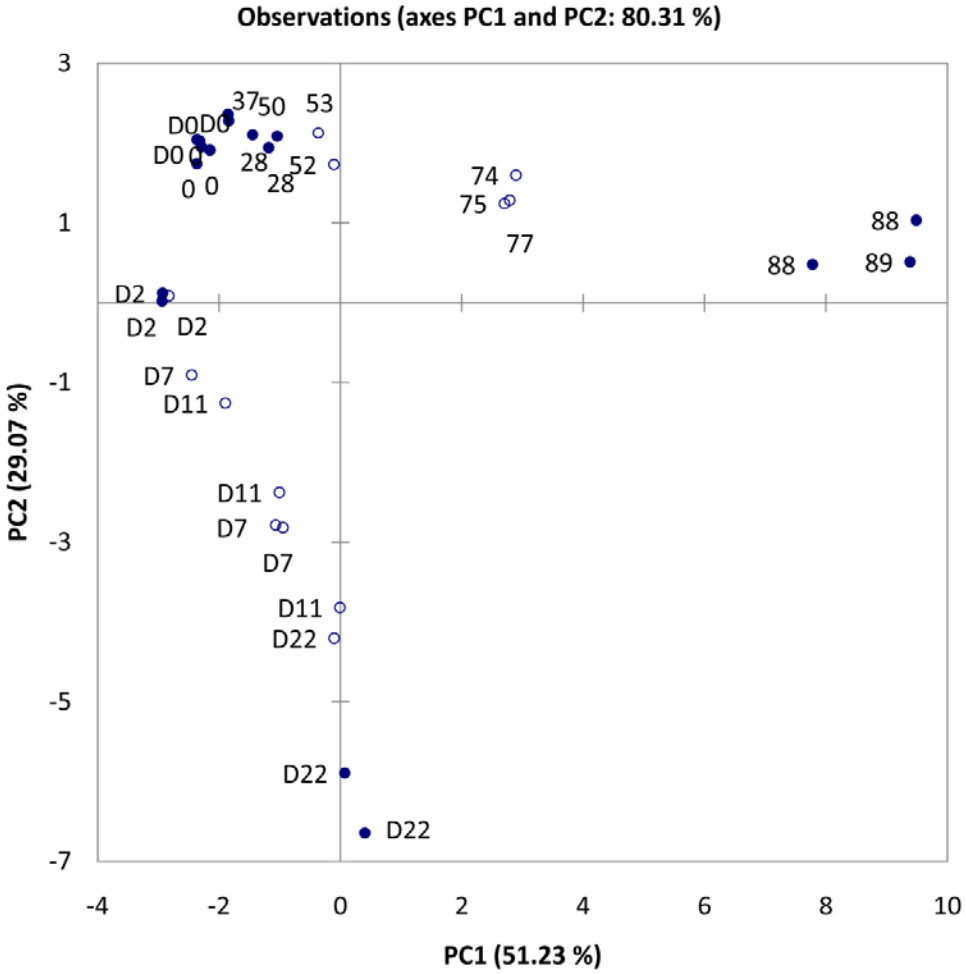


Figure 4-6 Plot of a data set that includes all chromatograms as projected into the first two principal components.

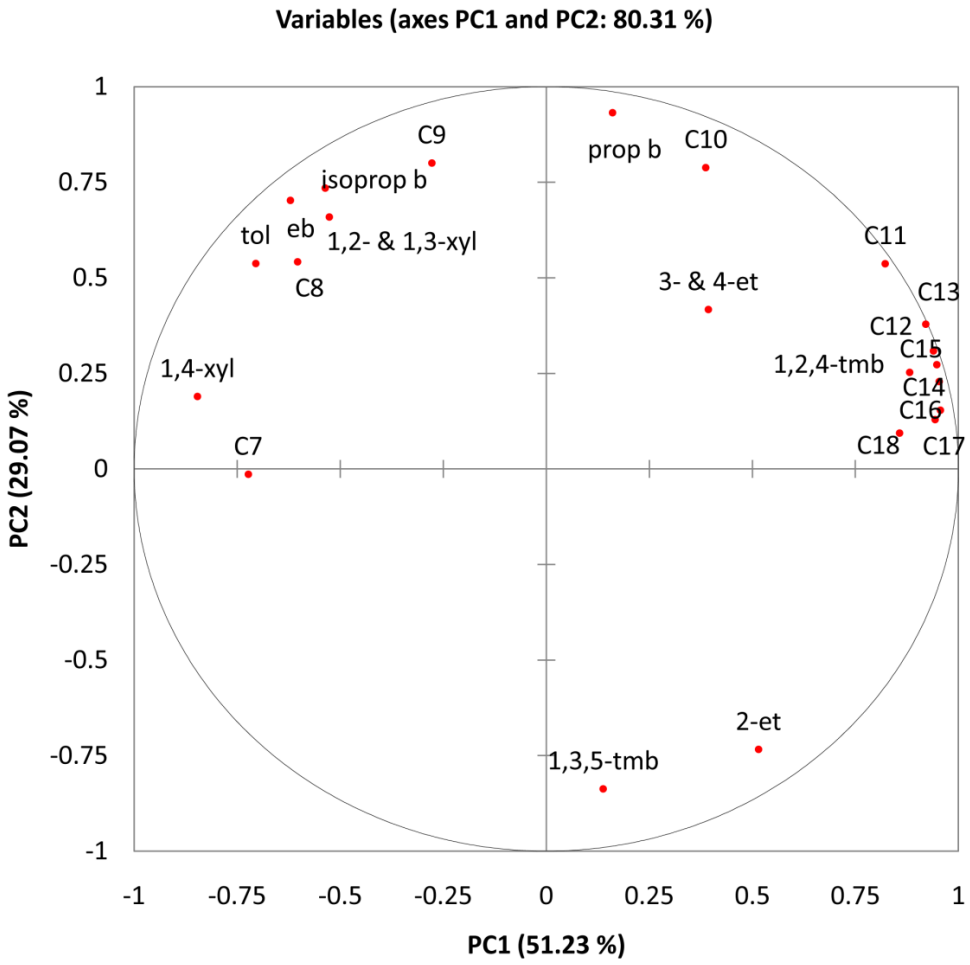


Figure 4-7 Plot of the factor loadings for a data set that includes all chromatograms.

As would be expected, the unweathered samples and the Day 0 degraded samples cluster closely together in the upper left quadrant of Figure 4-6. This region corresponds to samples that are fresh and undegraded as it is associated with low molecular weight n-alkanes and aromatics. The observations then rapidly diverge depending upon the process they undergo. The projections of the weathered samples move away from the lighter n-alkanes and aromatics (i.e., n-C_8 and toluene) and move toward the heavy n-alkanes and

aromatics (i.e. n-C_{14} and 1,2,4-trimethylbenzene). However, these heavier compounds are also susceptible to microbial degradation. Hence, degraded samples rapidly move away from all gasoline components and move toward aromatics that are the least susceptible to microbial degradation (1,3,5-trimethylbenzene and 2-ethyltoluene).

4.3.2 Field Studies

According to ASTM 1618, gasoline is identified based upon the presence of the C_3-alkylbenzenes (in ratios comparable to a standard gasoline sample) which include 3-ethyltoluene, 4-ethyltoluene, 2-ethyltoluene, 1,3,5-trimethylbenzene, and 1,2,4-trimethylbenzene [1]. However, since propylbenzene is a C_3-alkylbenzene and is included in the so-called "castle group" of chromatographic peaks [7], it will also be discussed. Other aromatic compounds of note in gasoline include toluene as well as the C_2-alkylbenzenes, known as the "three musketeers": ethylbenzene, m- & p-xylene, and o-xylene. Although these compounds are easily identified in a fresh gasoline sample, they are also the first to become significantly weathered by a fire.

One of the first distinctions that must be made in this data set is the effect of the substrate from which the gasoline residues were recovered. Figure 4-8 shows the total ion chromatograms (TICs) of gasoline recovered from either soil or glass immediately after gathering the samples (0 days). The gasoline residue recovered from the glass sample shows significant weathering, observed by the lack of toluene and the C_2-alkylbenzenes in the TIC. Additionally, the abundance of the C_4-alkylbenzenes, naphthalenes, and other higher boiling compounds are significantly higher than the C_3-alkylbenzenes, indicating that the sample has suffered from extensive (~98%) evaporation. The abundance of toluene and the C_2-alkylbenzenes in the gasoline residue recovered from the soil suggests

that the gasoline was less than 75% weathered [60]. Our explanation for this behavior is that as the gasoline vapors on top of the soil burn, the liquid seeps down into the porous soil and therefore is somewhat protected from the heat of the fire. This phenomenon is well known in the area of fire debris analysis and can be a significant effect for any type of porous substrate (e.g., carpet padding). In contrast, glass is not porous and therefore the ignitable liquid residue recovered from the glass is significantly more weathered than the residue recovered from the soil. In addition, there was significant variability among the gasoline residues recovered from glass fragments due to differing exposure to the hottest part of the fire. Therefore, glass samples were not used for further statistical analysis.

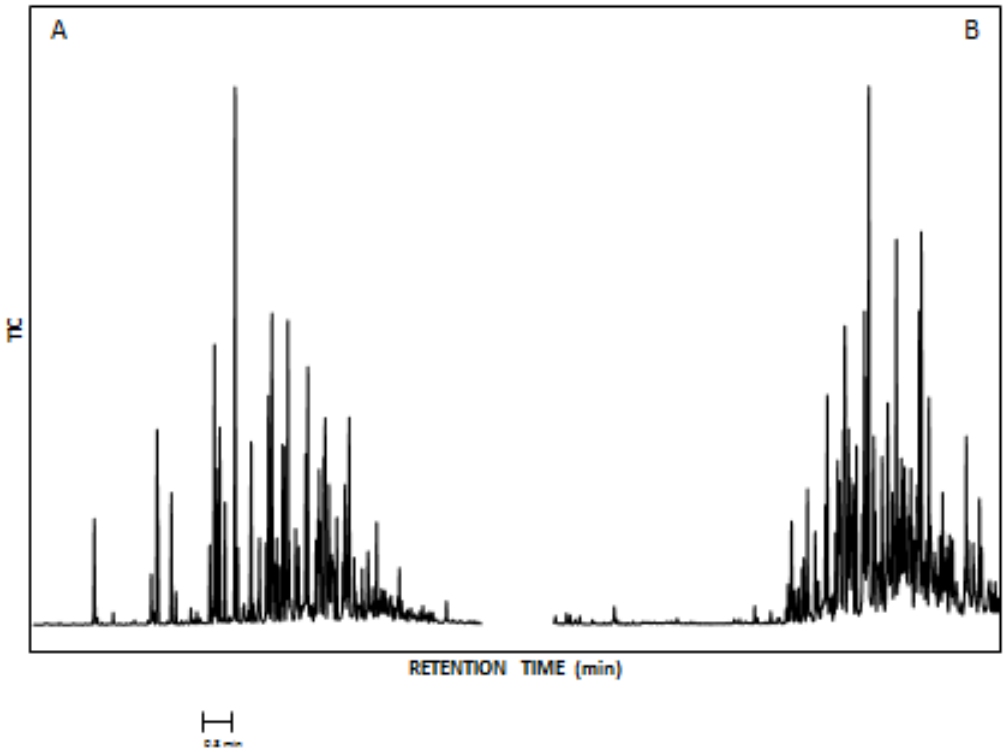


Figure 4-8 Substrate comparison of the recovery of gasoline from an incendiary device on: (A) soil and (B) glass after 0 days.

Figure 4-9 shows the microbial degradation of gasoline residues from the same type of incendiary device on two different soil types. The chromatographic profiles were very similar after 0 days, however, after 7 days differences were observed. For example, the relative amount of toluene and the C_2-alkylbenzenes decreased more in the lawn soil than in the potting soil. Additionally, the relative amount of propylbenzene (indicated by an asterisk) recovered from lawn soil is significantly less than that recovered from potting soil. After 60 days, the recovery is very low in both soils, but it was noticeably lower in the lawn soil. This observation is based upon an unexpected but useful measure of recovery that was noted in these chromatograms in the form of an early eluting peak that

was identified by a search of the MS database as a short chain aldehyde. This aldehyde was observed in all samples (including soil controls) and is conjectured to be emitted from the soil itself. While bacterial counts in the potting soil were similar to those in the lawn soil [61], the differences in degradation were greater in the lawn soil. The bacteria may simply have been more active in the lawn soil than the potting soil, perhaps because of the TOC, available nutrients, or moisture content that support physiological functions of the bacteria.

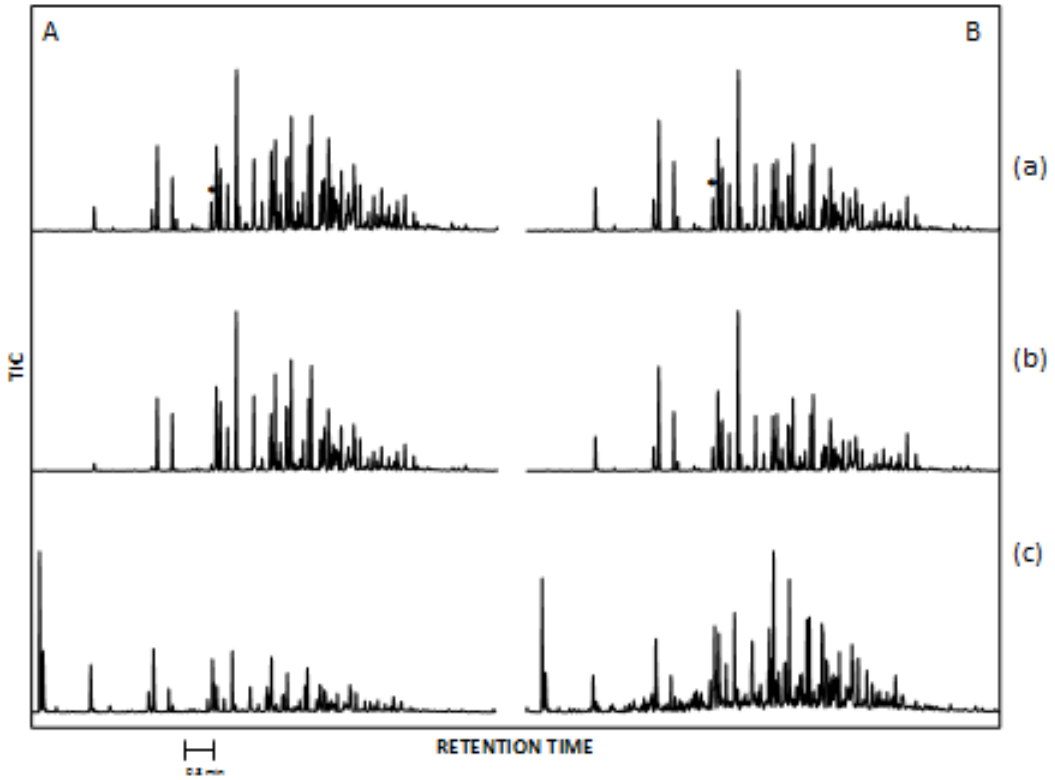


Figure 4-9 Soil type comparison of microbial degradation of gasoline from an incendiary device on: (A) Lawn soil and (B) Potting soil after: (a) 0 days, (b) 7 days, and (c) 60 days.

At this point, PCA was applied to this data set. The observations plot (Figure 4-10) describes how the average factor scores are projected into a two dimensional space for all

of the chromatograms associated with the soil type comparison. The projection of the data is roughly the same after 0 days, as would be expected given the similar chromatograms. Inspection of the factor loadings indicates that the variables that are most associated with day 0 samples are the n-alkanes between C_9 and C_{15} (Figure 4-11). These compounds have been shown to be the most susceptible to degradation; hence, they are expected to be more prevalent in fresh/non-degraded samples. Beyond 0 days, the data begins to "wander" across the two-dimensional scores plot. For example, the two soil types begin to diverge after 2 days and eventually follow completely different trajectories after 11 days. The samples reach their maximum degradation points at 60 days, where there is a clear difference in the projection of lawn soil and potting soil samples within the factor scores plot. Overall, these results suggest that the rate and degree of degradation of ignitable liquid residues does depend upon the type of soil to which it has been exposed.

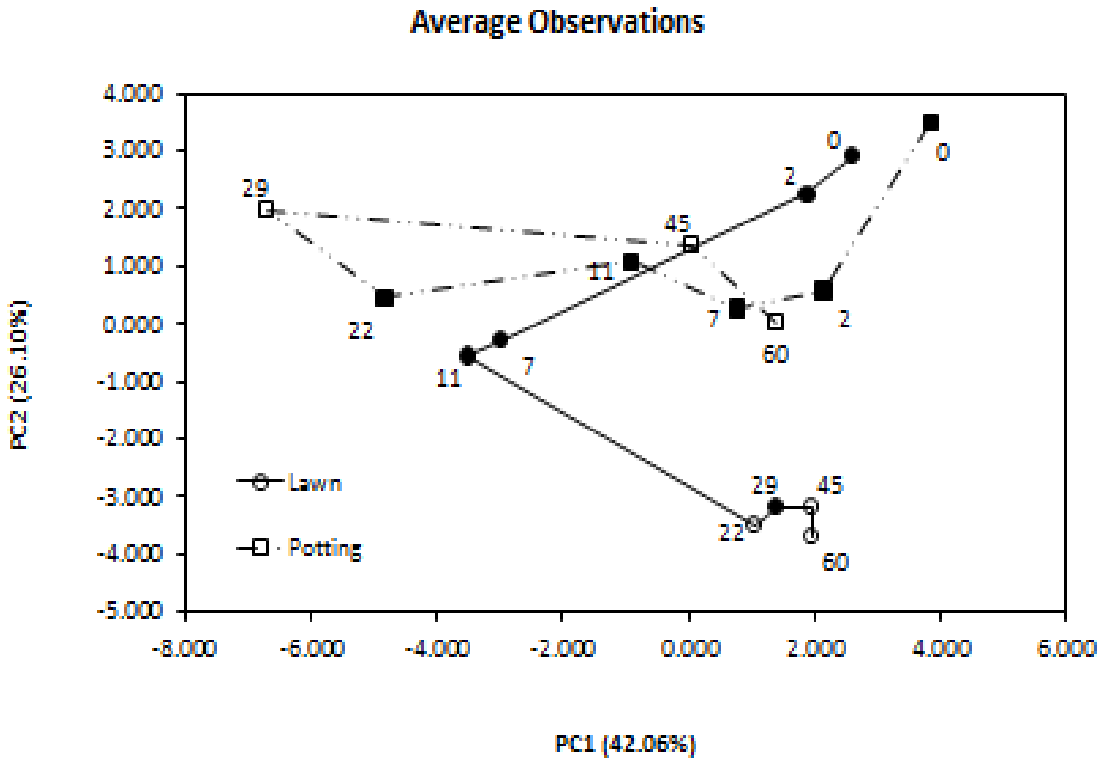


Figure 4-10 Averaged factor scores for the soil type comparison of microbial degradation of gasoline from an incendiary device on lawn soil and potting soil over 60 days.

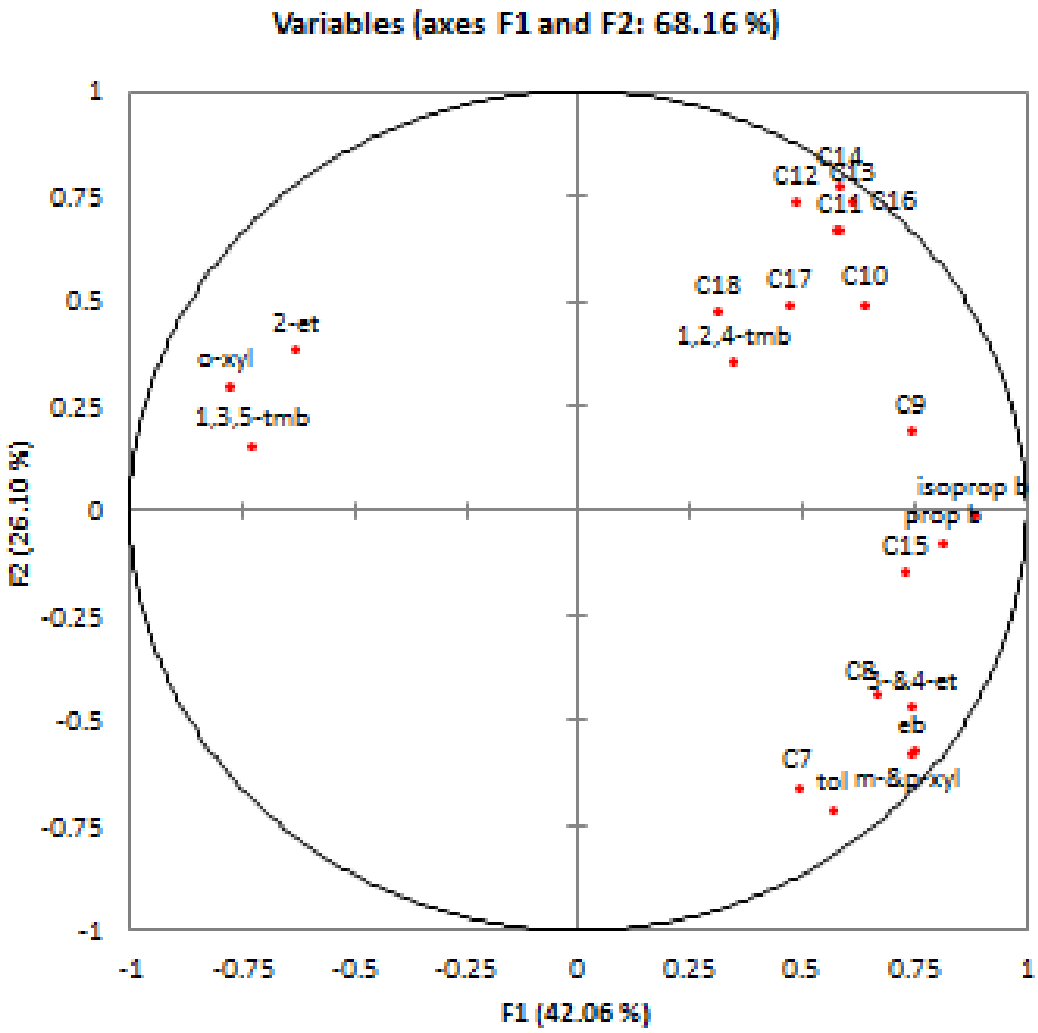


Figure 4-11 Factor loadings for the soil type comparison of microbial degradation of gasoline from an incendiary device on lawn soil and potting soil over 60 days.

The season may also have a profound impact on the microbial degradation of ignitable liquids, as the population of various bacteria can vary from season to season. Therefore gasoline residues recovered from an incendiary device on the same lawn soil during either summer or winter was analyzed over time. Figure 4-12 illustrates that the recovery of the gasoline residue (as seen relative to the soil aldehyde peak) is significantly lower in the winter sample than the summer soil. Additionally, the change in

the ratio of the C_3-alkylbenzenes appears to be more drastic over 60 days for the winter soil compared to the summer soil. One influencing factor could be the water content, although this was not measured. Water content plays an important role in biological activity. A moister soil supports bacterial growth than a drier soil. The summer soil may have been drier due to higher temperatures allowing water to evaporate out of the soil. It is also important to note that both soils, while collected during different seasons, were stored in sealed paint cans in the laboratory at room temperature. Storing samples at room temperature may have impacted the growth rate of bacteria which might also explain why the gasoline residues showed greater degradation in the winter soil than in the summer soil.

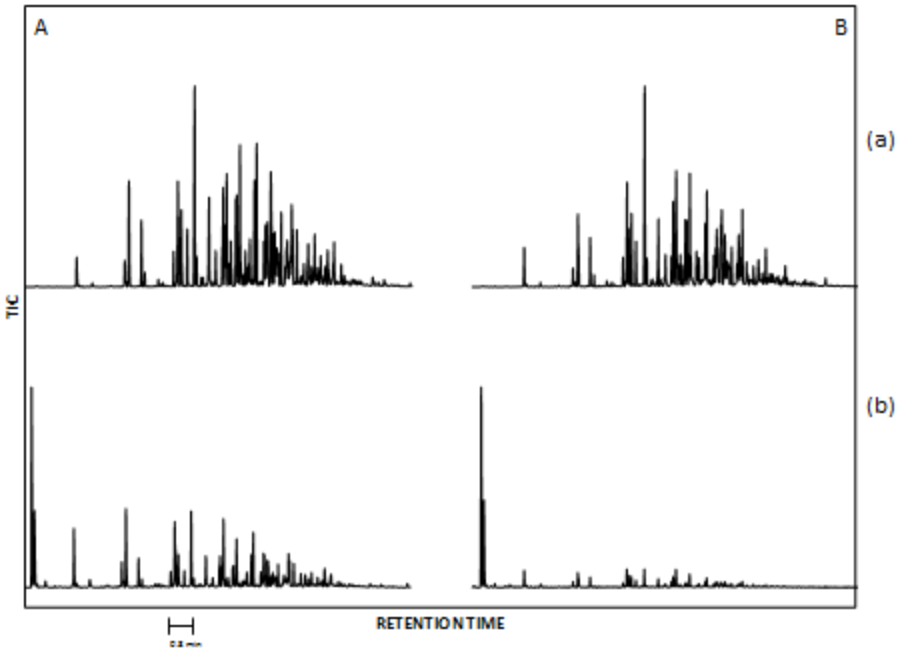


Figure 4-12 Seasonal comparison of microbial degradation of gasoline from an incendiary device on lawn soil during: (A) summer and (B) winter after: (a) 0 days and (b) 60 days.

While minor seasonal differences in microbial degradation may not be realized by visual inspection, PCA can be very useful in revealing these trends. For example, even though there is some separation in PC3, day 0 samples are projected in roughly the same 2D space (Figure 4-13). Many of the higher boiling n-alkanes as well as 1,2,4-trimethylbenzene are positively correlated with the first factor (F1) of the factor loadings as shown in Figure 4-14, suggesting that these compounds contribute significantly to both winter and summer day 0 samples. The samples follow a similar trajectory with slight differences in the degradation rate up to 22 days; at which point they split and a 180° difference in their trajectories is observed (Figure 4-13). The third principal component in this case is very helpful in observing the minor differences between the winter and summer soil samples. For example, the day 22 samples are practically overlapping in two dimensions, but there is some separation in the third dimension. The third dimension is illustrated by using filled and open data points. Open data points are correlated to PC3 in the negative direction and filled data points are correlated to PC3 in the positive direction. After 60 days, the factor loadings suggest that ethylbenzene and *m*- & *p*-xylene contribute most significantly to the summer sample, while these compounds are less significant in the winter sample (Figure 4-14).

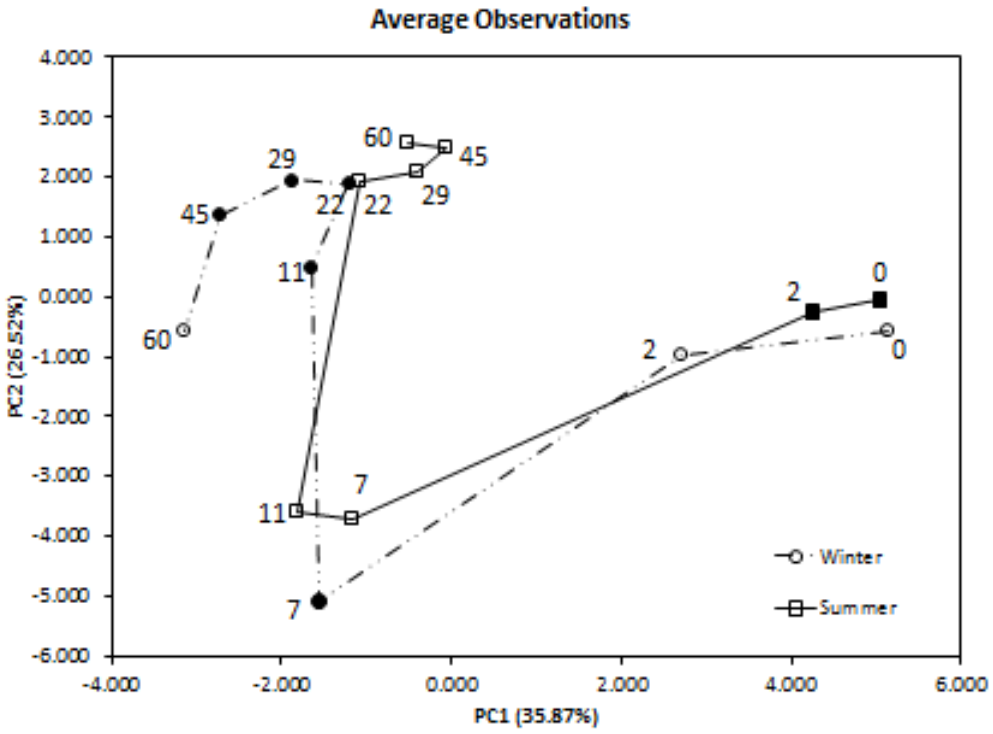


Figure 4-13 Averaged factor scores for the seasonal comparison of microbial degradation of gasoline from an incendiary device during summer and winter over 60 days.

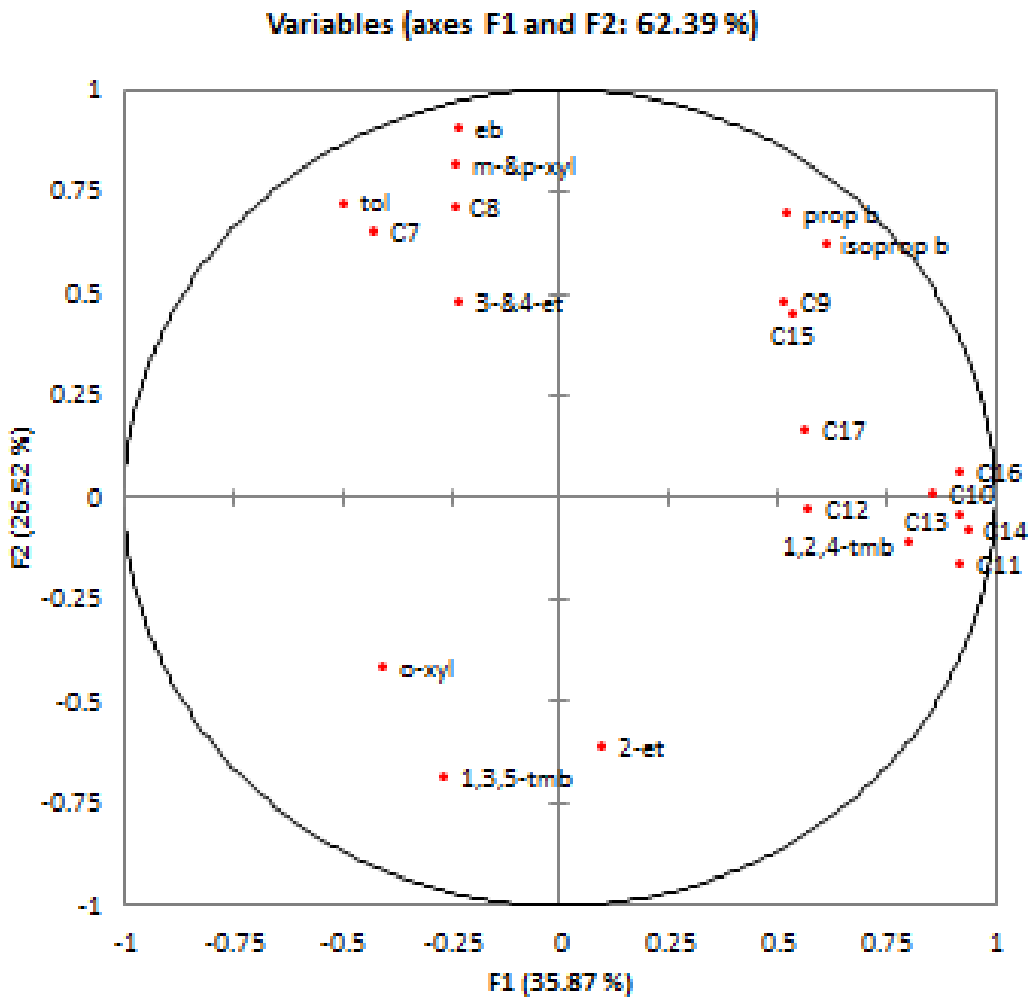


Figure 4-14 Factor loadings for the seasonal comparison of microbial degradation of gasoline from an incendiary device during summer and winter over 60 days.

A comparison of the volume of gasoline exposed to the soil was also tested by using two bottle sizes for the incendiary device to show that the amount of gasoline exposed to the bacteria may impact the chromatographic profile of degraded gasoline. After 7 days, there is a greater loss of the C_2-alkylbenzenes compared to the C_3-alkylbenzenes in the sample where a larger volume was used (Figure 4-15). The ratio of the C_3-alkylbenzenzes for both samples is quite similar after 0 days, however a

significant difference is observed after 7 days. In the wine bottle sample 1,3,5-trimethylbenzene appears to be decreasing compared to 3- and 4-ethyltoluene. In particular, the ratio between 3- and 4-ethyltoluene and 1,2,4-trimethylbenzene in the wine bottle sample is significantly larger than the that for the beer bottle sample. Additionally, the ratio of 1,3,5-trimethylbenzene and 2-ethyltoluene is smaller for the wine bottle sample than that for the beer bottle sample. The ratios of the C_3-alkylbenzenes are also significantly different between the two samples after 22 days, particularly the ratio between 2-ethyltoluene and 1,2,4-trimethylbenzene, and the ratio of 3-and 4-ethyltoluene compared to 1,3,5-trimethylbenzene. Finally, the ratio of the C_3-alkylbenzenes in the beer bottle sample did not appear to change significantly from 22 days to 60 days, but a loss of 2-ethyltoluene compared to 1,3,5-trimethylbenzene was observed for the wine bottle sample.

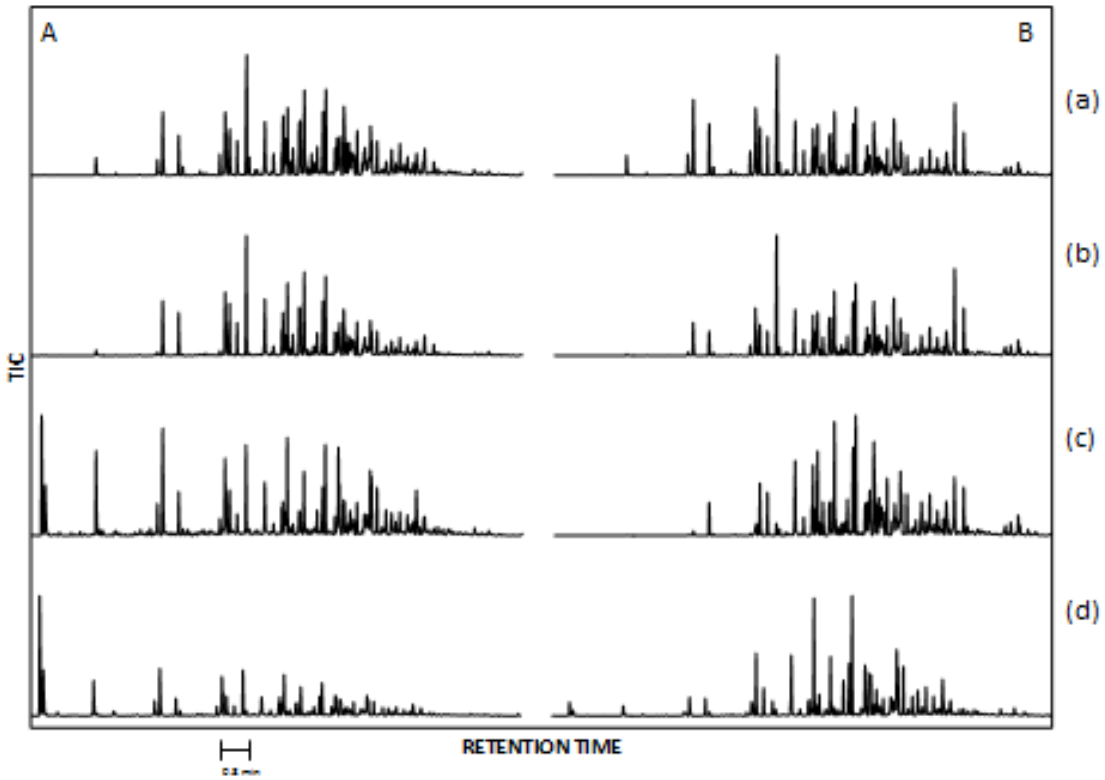


Figure 4-15 Volume comparison of microbial degradation of gasoline from an incendiary device on Lawn Soil from a: (A) beer bottle and (B) wine bottle after: (a) 0 days, (b) 7 days, (c) 22 days, and (d) 60 days.

PCA was then performed to show differences in microbial degradation between the amounts of gasoline exposed to the soil. As shown in Figure 4-16, the samples are projected in the same space at day 0, but they quickly take different paths after 7 days. The factor loadings suggest that at 0 days *m*- & *p*-xylene and 3-and 4-ethyltoluene contribute most significantly to both samples (Figure 4-17). After 60 days, the lower boiling n-alkanes, toluene, and ethylbenzene contribute more significantly in the beer bottle sample, while higher boiling n-alkanes and 1,3,5-trimethylbenzene contribute more significantly in the wine bottle sample.

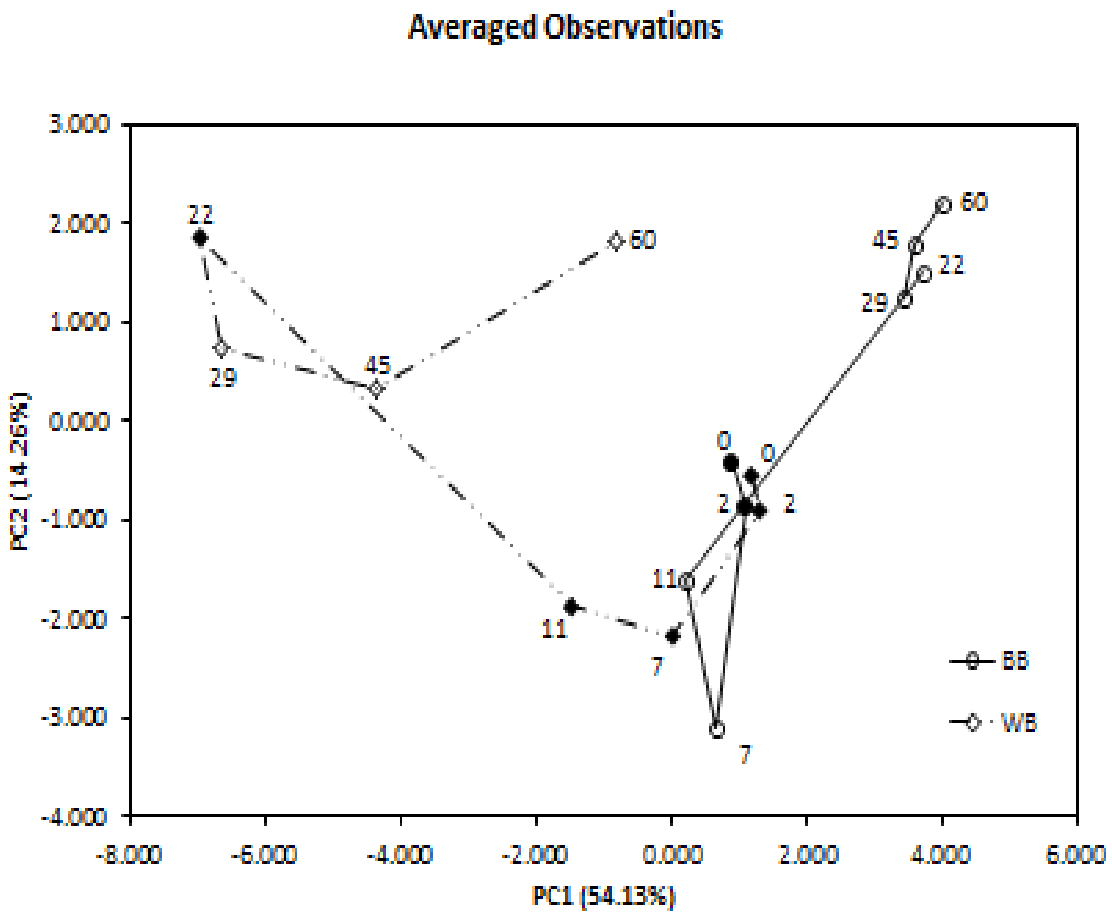


Figure 4-16 Averaged factor scores for the volume comparison of microbial degradation of gasoline from a beer bottle and a wine bottle over 60 days.

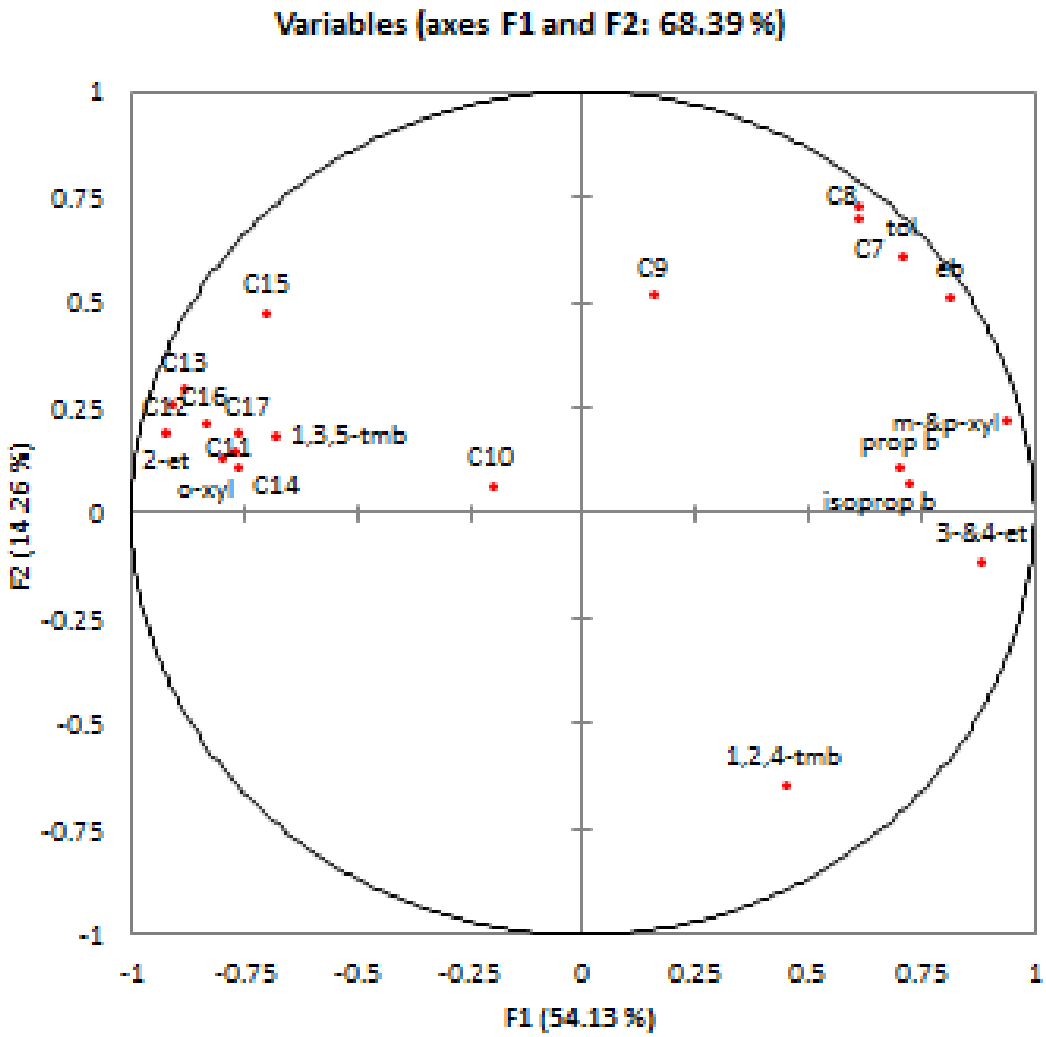


Figure 4-17 Factor loadings for the volume comparison of microbial degradation of gasoline from a beer bottle and a wine bottle over 60 days.

4.4 Conclusions

4.4.1 Laboratory Study

Weathering and microbial degradation of ignitable liquid residues are two separate processes that can be readily distinguished by PCA. Compounds with boiling points less than ~ 160 ^{o}C are the most susceptible to weathering. In contrast, long chain alkanes and lower substituted aromatics are susceptible to microbial degradation,

regardless of boiling point. In particular, PCA revealed that 1,3,5-trimethylbenzene and 2-ethyltoluene were not only resistant to weathering, but they were the least susceptible to microbial degradation.

4.4.2 Field Studies

In particular, glass samples exhibited the loss of low boiling compounds while high boiling compounds were retained. However, in soil samples, even though the gasoline was initially weathered from the fire, the ignitable liquid residue suffered most from microbial degradation, which showed the loss of both low boiling (e.g., *m*- & *p*-xylene) and high boiling compounds (e.g., 3-ethyltoluene).

Additionally, microbial degradation appears to vary depending on the type of soil. After 60 days on soil, the gasoline was significantly more degraded from exposure to lawn soil than when exposed to potting soil. Potting soil is a manufactured product designed to contain nutrients such as nitrogen, carbon, and other compounds for optimal growth of plants. Native soils may likely contain a variety of nutrients that not only provide nutrients for plants, but also allow for optimal growth of bacteria. Therefore, the bacterial counts in native soil types may differ significantly from potting soil. Higher bacterial counts in lawn soil would explain why degradation in lawn soil was more apparent. In addition, PCA results also indicate that the rate and degree of degradation is impacted by the type of soil to which the ignitable liquid was exposed.

The degree of degradation is also affected by the season in which the sample was collected. It was expected that microbial degradation would be less pronounced in a winter sample as compared to a summer sample, due to a decreased population of microbes. However, the opposite was observed. This could be explained by the fact that

summer soil contains more available nutrients that are readily metabolized by bacteria, which would reduce the demand for hydrocarbons as a source of energy.

The volume of ignitable liquid may also impact how quickly changes in the chromatographic profile are noted. While the ratios of the relevant peaks in gasoline recovered from the wine bottle sample are higher compared to the aldehyde peaks, the ratio of the C_3-alkylbenzenes are significantly distorted. Whereas in the beer bottle sample, the ratio of the C_3-alkylbenzenes are not significantly altered but the overall recovery is low compared to the aldehyde peaks.

Microbial degradation is becoming a well-known phenomenon amongst the forensic community; however previous works have focused on the study of “neat” liquids, which are not realistic samples in fire debris analysis. This work seeks to broaden the understanding of microbial degradation of ignitable liquids as well as the application of chemometrics to the analysis of fire debris.

CHAPTER 5. BALL STATE UNIVERSITY MICROBIAL DEGRADATION STUDIES

5.1 Introduction

The number of incendiary fires in the U.S. averages approximately 210,300 every year, which comprises about 13% of the total of all reported fires, according to FEMA's Topical Fire Report Series [62]. On an annual basis, incendiary fires claim 375 lives, injure over one thousand people, and cause approximately $1 billion in direct property damage [62]. In many cases, the arsonist uses an ignitable liquid to accelerate the fire. Gasoline is the most commonly used ignitable liquid as it is readily accessible, inexpensive and ignites easily [7]. Gasoline and other ignitable liquids are classified according to the American Society of Testing and Materials (ASTM) guidelines by their boiling point range and chemical composition [1]. In practice, a forensic chemist will use various extraction methods coupled with gas chromatography/mass spectrometry (GC/MS) to determine if an ignitable liquid residue (ILR) is present in a fire debris sample. The ILR will then be classified according to ASTM guidelines [7, 32].

Media rich in organic matter such as soil provides a rich source of carbon and typically contains substantial quantities of active bacterial biomass. Since ignitable liquids are composed of a range of hydrocarbons, they may be suitable as a carbon source by bacteria. Such transformations are problematic for fire debris analysis as samples are often stored for many weeks at room temperature before they are analyzed

due to case backlog and lack of cold storage. As a result, selective loss of hydrocarbon species due to bacterial metabolism can occur, making the identification and classification of ignitable liquid residues difficult or even impossible. For example, five specific C_3-alkylbenzenes (3-ethyltoluene, 4-ethyltoluene, 1,3,5-trimethylbenzene, 2-ethyltoluene and 1,2,4-trimethylbenzene) must be identified in a sample in order to determine if residues of gasoline are present. Furthermore, because these compounds also occur in other materials, they must be present in relative amounts that are similar to that of a gasoline standard [1]. Among the serious consequences of microbial degradation are the selective losses of some of these compounds and/or changes in the ratios of these compounds in a gasoline sample. While other fire debris substrates such as carpeting and bedding can contain a substantial amount of bacteria, microbial degradation of ignitable liquids in samples containing these substrates has not been reported. This is perhaps because common bacteria found in these substrates do not metabolize hydrocarbons found in ignitable liquids.

Several factors affect bacterial numbers and activities in soil including soil type and season. Chemical and physical characteristics of soils including pH, nitrogen level and phosphorus content will vary, as do soil physical properties (e.g., texture). In turn, varying populations of bacteria may impact the degree of microbial degradation observed in fire debris samples containing soil.

Previous work has demonstrated that bacteria readily degrade normal alkanes (e.g. decane) and lesser substituted alkylbenzenes (e.g., toluene, ethylbenzene, propylbenzene) while more highly substituted alkylbenzenes (e.g., 1,2,4-trimethylbenzene) and highly branched alkanes are more resistant to microbial attack [32, 54, 55]. While treatment of

hydrocarbon-contaminated soils by bacteria is a well-known phenomenon in the environmental engineering community [9, 34, 38, 50-53, 63-66], microbial processes are not well understood in forensic science. This phenomenon likely varies with soil type and over different seasons as soil chemical properties, temperature and moisture status may impact heterotrophic bacteria.

The overall objectives of this study were to assess the degradation of a common ignitable liquid (i.e., 87 octane gasoline) in soil as affected by soil type and season of year. The focus of this paper will be upon the effect of soil type and season, to include: (1) analysis of GC/MS data from gasoline added to three different soils over all four seasons; (2) identification and quantification of bacterial populations present in the study soils; and (3) quantification of organic and inorganic compounds present in the study soils.

5.2 Materials and Methods

5.2.1 Soil Chemical Analysis

Soil material was obtained from an agricultural field (Pella clay), a residential property (Miamian sandy clay), and a brownfield site (Urban land/Wawaka-Miami complex clay) in central Indiana. Soil material was collected from the surface 0-20 cm of each site using a stainless steel sampling probe. The soil was composited in the field, and air-dried and sieved (< 2 mm mesh) in the laboratory.

Particle size distribution of the soils was determined using the hydrometer method [67]. Total organic carbon (TOC) and total nitrogen (N) were analyzed on a Perkin Elmer Series II CHNS/O Analyzer 2400 (Shelton, CT). Acetanilide was the standard used. Soil

pH was determined using a 1:2 (w:v) solids:deionized water slurry with an AB15 Accumet pH meter.

Soil nitrate (NO_3) concentrations were measured using Szechrome reagents [68] in a BioteK PowerWave XS2 microassay system. Soil ammonium (NH_4) concentrations were determined by the method of Sims et al. which uses a modified indophenol blue technique [69]. The method was adapted for the BioteK PowerWave system. Soil extractable P was determined by the Bray-1 method [70]. Soil K was extracted with neutral 1.0 M ammonium acetate and analyzed using atomic emission spectrophotometry (Perkin Elmer AAnalyst 2000). Extractable metal (Cd, Cr, Fe, Zn, Pb) concentrations were determined by extraction with 5 mM DTPA (diethylenetriaminepentaacetic acid) with 10 mM $CaCl_2$, pH adjusted to 7.3. Briefly, the method involved mechanical shaking (120 osc./min. for 2 h) of 5 g soil with 25 ml of 5 mM DTPA in acid-washed Nalgene® bottles. The suspension was filtered through Whatman No. 2 filter paper and analyzed for Cd, Cr, Fe, Zn and Pb using flame atomic absorption spectrophotometry (Perkin Elmer AAnalyst 2000). For the above analyses, there were four replicates of each sample.

5.2.2 Soil Microbiological Analyses

Populations of total culturable bacteria were determined in each soil type using the standard plate count [71] on Plate Count Agar (PCA, Teknova, Hollister, CA). Soilborne actinomycetes were enumerated on Actinomycete Isolation Agar (Sigma-Aldrich, St. Louis, MO) and yeasts and molds were quantified using Sabouraud Dextrose agar (Fisher Scientific, Waltham, MA). Six replicates of each soil type colony counts were averaged following 48h incubation of all inoculated plates.

For the genetic identification of bacteria, DNA was obtained from 3-5 g soil samples using a commercial system (MoBIO, Solana Beach, CA) and quantified spectrophotometrically. Real-time PCR was carried out in a Smart Cycler II (Cepheid, Sunnyvale, CA). Extracted DNA (1 µg) was added to real-time SYBR Green™ Supermix (Quanta Biosciences, Gaithersburg, MD); a no-template contamination control was analyzed for each sample/primer set, as well as positive control specimens consisting of genomic DNA from ATCC (Manassas, VA) type strains or other reference strains of *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, and *Flavobacterium* (Table 5-1). All PCR primers were designed with the software analyses options available through the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) (www.ncbi.nlm.nih.gov/BLAST), which allows for sequences to be screened for nonspecific annealing frequencies and non-target homology determination. Internal standard primer targets in each case were the highly conserved prokaryotic gyrase subunit B gene, *gyrB* [72]. Each primer pair was tested on all non-target strains to ensure appropriate specificity and eliminate the appearance of false-positive amplification signal. Cycling conditions were 10 min. at 95°C, followed by 40 three-step cycles of 15 s at 95°C , 1 min. at 55°C and 1 min at 72°C, with fluorescence acquisition monitored at the end of each cycle.

Table 5-1 ATCC reference strains and PCR primers used in the rDNA-based quantification aspect of this study.

Strain designation	5'→3' primer sequences	Reference
Acinetobacter calcoaceticus 346[1]	TAC GCA GGG TAA TGA ATC AA TCC GTG TCT CAG TAC CAG TG	Chang et al., 2005
Alcaligenes faecalis subsp. *faecalis* ATCC 8750[2]	CAT CCC GCG GTG TAT GAT GAA TCT GAC ATA CTC TAG CTC GG	Phung et al., 2012
Arthrobacter globiformis 607[1]	GTC GCG TCT GCT GTG AAA GC TTT AGC CTT GCG GCC GTA CT	Crocker et al., 2000
Bacillus cereus ATCC 14579[2]	AGA GTT TGA TCC TGG CTC AG TAC GGC TAC CTT GTT ACG ACT T	Bavykin et al., 2004
Flavobacterium capsulatum 315[1]	TAC TCG CAG AAT AAG CAC CG GTA TCT AAG TTC CCG AAG GC	GenBank Accession M59296
Pseudomonas fluorescens 13525	GGTCTGAGAGGATGATCAGT TTAGCTCCACCTCGCGGC	Widmer et al., 1998

[1]Reference strain obtained from Presque Isle Cultures, Erie, PA
[2]Reference strain obtained from American Type Culture Collection, Manassas, VA

Standard curves to determine number of copies of target genomes for each bacterial genus were constructed using quantified bacterial templates obtained from each reference strain 1:10 serially diluted in nuclease-free water to 10^{-6} (each diluted in triplicate) and subjected to amplification as described above. Bacterial template concentrations were converted to amplicon (PCR product) copies by multiplying the mean grams of DNA purified for each set of extraction replicates by 6.02 X 10^{23}, and dividing that product by the product of the respective amplicon length in base pairs X 650

Daltons. Resulting plots depict the number of amplicon copies as a function of respective cycle threshold (Ct) values.

5.2.3 Microbial Degradation Studies

For each soil type, eight sample time points were prepared in triplicate by spiking 20 μL of commercial unleaded gasoline (87 octane) onto ~100 g soil in a clean but non-sterile quart-size paint can. The samples were sealed and stored for 0, 2, 4, 7, 11, 15, 22, and 30 days. On each specified day, the samples were extracted using passive headspace adsorption-elution (a popular and widespread extraction technique for fire debris) [5]. In this method, one third (~7 x 9 mm^2) of a charcoal strip (Albrayco Technologies, Cromwell, CT) was placed in each can and suspended in the headspace on a pre-baked (at 85°C) paper clip using nylon string. The re-sealed cans were heated at 85 °C for 4 h. After cooling, the charcoal strips were removed and extracted with 400 μL of pentane with vortexing for ~1 min. Samples were then analyzed by GC-MS (Agilent 6890 GC with an Agilent 5975 MSD) using a standard method for fire debris analysis, which includes a 1 μL injection volume, 20:1 split ratio, inlet temperature of 250 °C, flow rate of 1 mL/min (helium), a DB-5 30 m x 0.25 mm x 0.25 μm column, initial column temperature of 40 °C held for 2 min, temperature ramp of 20 °C/min, final temperature of 280 °C held for 3 min, solvent delay of 2 min, MS scan of 40-300 m/z, MS quad temperature of 150 °C and an MS source temperature of 230 °C [32, 54, 55].

5.2.4 Data Analysis

Each analyte (Table 5-2) was identified based on comparison to the retention time and mass spectrum of an authentic standard. A comparison was also made to the National

Institute of Standards and Technology (NIST) mass spectral database. The peak areas from the summed extracted ion profiles (alkane: m/z 57, 71, 85, 99; aromatic: m/z 91, 120; benzaldehyde: m/z 77, 106) were exported into Microsoft Excel from the Xcalibur data analysis software (Thermo Scientific, Hanover Park, IL). Extracted ion profiles are employed in fire debris analysis in order to filter out interfering signals that could otherwise impede the classification of the ignitable liquid. The peak areas of the compounds listed in Table 1 were normalized and then autoscaled. Normalization corrects for differences in overall instrument response and autoscaling allows the variance for each variable to be weighted equally [59]. XLSTAT (AddinSoft), an add-in for Microsoft Excel, was used to run Principal Component Analysis (PCA) on the autoscaled data for each soil type. PCA is a data reduction technique that allows for the visualization of samples in a two-dimensional plot despite the fact that the samples are described by many variables. For example, PCA has been used to discern differences in the relative chemical composition of samples that underwent evaporation versus microbial degradation [55].

Table 5-2 Compounds of interest which were monitored in the microbial degradation of gasoline in soil.

Compound	Variable Label	bp (°C)
Heptane	C7	98
Toluene	tol	111
Octane	C8	125-127
Ethylbenzene	eb	136
p-xylene	p-xyl	138-139
o-xylene	o-xyl	143-145
Nonane	C9	151
isopropylbenzene	isoprop b	152-154
Propylbenzene	prop b	158
3-&4-ethyltoluene	3-&4-et	158-159
1,3,5-trimethylbenzene	1,3,5-tmb	163-165
2-ethyltoluene	2-et	164-165
1,2,4-trimethylbenzene	1,2,4-tmb	168
Decane	C10	172-174
Undecane	C11	196
Dodecane	C12	216
Tridecane	C13	234
Tetradecane	C14	252-254
Pentadecane	C15	270
Hexadecane	C16	287
Heptadecane	C17	302
Octadecane	C18	317

5.3 Results and Discussion

5.3.1 Soil Chemical Analyses

The results of the soil chemical analyses are summarized in Table 5-3. All soil samples contained high percentages of clay (29.8 to 53.9%). Soil textures ranged from sandy clay to clay. These textures are typical for much of the northern two-thirds of the state of Indiana, which is overlain by substantial deposits of till from the Wisconsin glacial epoch [73].

Table 5-3 Selected chemical and physical properties of the study soils.

	Agricultural	Residential	Brownfield
pH	6.6	6.3	6.6
Total N, %	0.44	0.23	0.23
NO_3^-, mg/kg	60.9	40.4	26.3
NH_4^+, mg/kg	2.8	3.0	1.9
TOC*, %	1.0	1.0	0.9
Bray-1 P, mg/kg	5.3	137.3	5.3
Extractable K, mg/kg	122.8	154.5	74.0
Extractable metals, mg/kg			
Cd	0.32	0.43	0.54
Cr	0.01	0.16	0.11
Fe	48.5	39.5	18.0
Zn	13.5	24.2	22.7
Pb	11.8	12.7	497.0
Particle size analysis			
Sand, %	28.8	46.8	29.6
Silt, %	20.6	12.7	16.5
Clay, %	50.6	40.5	53.9
Texture	clay	sandy clay	clay

*TOC = total organic carbon.

Soil pH ranged from 6.3 (residential) to 6.6 (agricultural and brownfield). Total soil N ranged from 0.23 mg/kg (residential and brownfield) to 0.44 mg/kg (agricultural)

(Table 3). The brownfield soil was lowest in NO_3 (26.3 mg/kg), whereas the agricultural soil had the highest quantity (60.9 mg/kg). Soil NH_4 levels were similar across soil type, ranging from 1.9 mg/kg (brownfield) to 3.0 mg/kg (residential). Soil TOC was similar across treatments with values ranging from 0.9 to 1.0 % (Table 5-3).

Levels of extractable Cd, Cr, Fe and Zn were all within range for non-contaminated soils (Table 5-3). In the brownfield soil, extractable Pb levels measured 497 mg/kg. An upper limit for Pb content of a normal soil is approximately 70 mg/kg [74]. The levels of Pb in soils that are toxic to soil microorganisms and plants are a function of species, Pb concentration and soil factors (e.g., pH, fertility status, presence of other toxins); thus, threshold toxicity levels will vary. Soil Pb levels considered toxic to biota have ranged from 100 to several thousand mg/kg [75, 76].

5.3.2 Soil Microbiological Studies

Populations of recoverable aerobic chemoheterotrophic bacteria were analyzed from each soil treatment using SPC on PCA. Table 5-4 reveals the average density was not significantly different ($p > 0.05$) across soil types at 3.8×10^5 cfu/g. Likewise, detectable actinomycetes remained stable as well (average = 6.9×10^5 cfu/g, and were not significantly different for any soil treatment (($p > 0.05$). Total recoverable fungal counts similarly revealed no significant differences among treated soils (1.1×10^5 cfu/g). However, since culture-based methods reveal only a subset of a microbial population in any given sample, PCR was used to determine comparative levels of selected bacterial genera across soil types in order to deduce what roles if any each genus may play in degradation of the ignitable liquid used here.

Table 5-4 Microbiological plating-based results. Values shown represent mean colony counts of eight replicates, which were not significantly (p>0.05) different across the sampling times (Fall, Winter, Spring, & Summer). PCA= Plate count agar for total chemoheterotrophic bacteria; ACT = actinomycete agar, for soilborne actinomycetes; SDA = Sabouraud dextrose agar for total molds and yeasts.

Media	Agricultural	Residential	Brownfield
PCA	$5.02x10^5$	$2.54x10^5$	$6.86x10^5$
ACT	$4.62x10^5$	$7.1x10^5$	$1.38x10^6$
SDA	$2.28x10^5$	$4.48x10^4$	$1.24x10^5$

rDNA-based PCR detection was used to quantify total genome equivalents for representative bacterial genera demonstrating a previous history in the literature of chemical adulterant metabolism in soil environments [77-81]. Specifically, *Acinetobacter*, *Alcaligenes, Arthrobacter, Bacillus*, *Flavobacterium,* and *Pseudomonas* genera were analyzed here by qPCR using genus-specific PCR primers and standard curves generated with ATCC type strain DNA (Table 5-3). The calculated total genome equivalents of each bacterial genus is shown in Table 5-5. These qPCR results reveal that in all soil treatments, *Alcaligenes* spp. were consistently detectable at significantly higher levels ($p < 0.05$) than any other genus. *A. faecalis* has been reported to degrade the chlorinated insecticide endosulfan, found routinely in many soil types, water, and as residue on foods due to its widespread use in, and rapid transport through, the natural environment [82]. The possibility exists that levels of this and perhaps other species of *Alcaligenes* are present in the soils analyzed in our study due to some effect by this or a similar ubiquitous chlorinated derivative. Calculated genome copies of each bacterial group

using rDNA-specific PCR revealed densities many orders of magnitude above recovered bacterial densities on PCA. This is attributed to the fact that our DNA-based PCR is detecting template copies from both viable bacterial targets and from dead cells accumulated in the soil biomass. However, these numbers are nevertheless still quite revealing on relative levels of each genus across soil types, and will be even more interesting in further examination of these soils when compared seasonally.

Table 5-5 qPCR-based determination of genome copies per gram of soil from each bacterial genus in this study. Values are the mean value from triplicate samples analyzed using SYBR Green-based standard curves as described in Materials and Methods.

Bacterial Genus	**Agricultural**	**Residential**	**Brownfield**
Acinetobacter	$4.32x10^{16}$	$3.12x10^{16}$	$1.31x10^{17}$
Alcaligenes	$7.29x10^{19}$	$2.37x10^{20}$	$2.26x10^{18}$
Arthrobacter	$4.135x10^{10}$	$1.636x10^{13}$	$1.396x10^{12}$
Bacillus	$4.06x10^{14}$	$5.12x10^{14}$	$3.36x10^{14}$
Flavobacterium	$1.8x10^{16}$	$4.53x10^{10}$	$4.21x10^{11}$
Pseudomonas	$1.624x10^{14}$	$1.682x10^{14}$	$2.56x10^{15}$

It is well known that DNA-based PCR detects both dead and viable bacteria, however, so in order to more accurately ascertain levels of only viable bacterial genera playing an active role in biodegradation, we targeted mRNA and quantified only bacteria actively transcribing their respective rDNA genes (Table 5-3) [83]. *Alcaligenes* appears at the highest viable density in residential soil, and the lowest in Brownfield soil. However, even in the latter, more industrially contaminated soil type, 80,000 copies/g of

Alcaligenes were detected suggesting these bacteria persist using an as-yet-undetermined physiological mechanism in the presence of lower NO_3 and higher Pb levels, and/or in the presence of chlorinated adulterants as noted above, an area of future research interest.

Based on DNA PCR results, residential soil exhibited the largest variation in levels of bacterial genera under study compared to other soil types, as well as the highest levels of bacteria overall. However, qRT-PCR results (Table 5-6) speak to a slightly different proportion of viable bacteria. *Pseudomonas* spp. were detected at the lowest levels in residential soil ($3.00x10^5$/g) while the spore-forming *Bacillus* spp and *Alcaligenes* were at the highest viable density ($3.06x10^{17}$ and $5.56x10^{13}$, respectively).

Table 5-6 qRT-PCR-based determination of specific mRNA transcript copies per gram of soil to ascertain viable cell densities from each bacterial genus in this study according to treated soil type. Values are the mean value from triplicate samples analyzed using SYBR Green-based standard curves as described in Materials and Methods.

Bacterial Genus	**Agricultural**	**Residential**	**Brownfield**
Acinetobacter	$5.30x10^1$	$1.16x10^2$	$1.27x10^6$
Alcaligenes	$1.43x10^7$	$5.56x10^{13}$	$8.23x10^4$
Arthrobacter	$6.84x10^7$	$5.32x10^{12}$	$4.00x10^8$
Bacillus	$1.80x10^{13}$	$3.06x10^{17}$	$2.61x10^{10}$
Flavobacterium	$3.35x10^{25}$	$7.68x10^{11}$	$1.72x10^3$
Pseudomonas	None detected	$3.00x10^5$	$2.37x10^5$

5.3.3 Microbial Degradation Studies

5.3.3.1 Soil Type Comparison

Microbial degradation of gasoline was observed in the residential, agricultural, and brownfield soils (Figs. 5-1 and 5-2). In all three soil types, n-alkanes were degraded in a similar fashion in that degradation is almost complete after 7 days. In fact, no peaks remained in the chromatograms by 15 days except those attributed to volatile aldehydes that are present in the headspace of all soil samples (Figure 5-1). In contrast, we noted differences in the ratios of the C_3-alkylbenzenes depending upon soil type (Figure 5-2). For example, all profiles appear nearly identical on day 0, but on day 2 ethylbenzene (peak 1) is significantly reduced in comparison to 3-ethyltoluene (peak 2) in the residential soil whereas in the agricultural and brownfield soils only minimal reduction is apparent. By 30 days the gasoline in residential and agricultural soils experienced the greatest microbial degradation while the gasoline in the brownfield soil experienced the least.

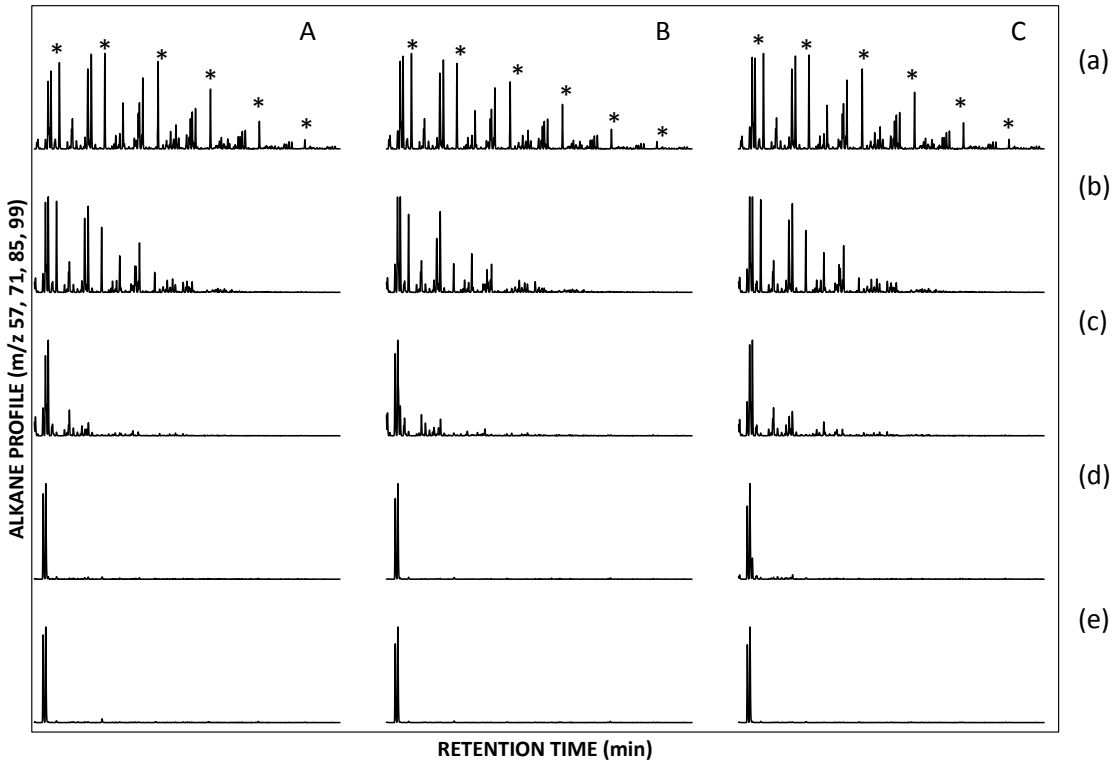


Figure 5-1 Alkane profile for the soil type comparison of microbial degradation of gasoline on: (A) agricultural soil, (B) residential soil, and (C) industrial soil over (a) 0, (b) 2, (c) 7, (d) 15, and (e) 30 days (Fall sampling). Peaks from the homologous series of n-alkanes are marked with an asterix.

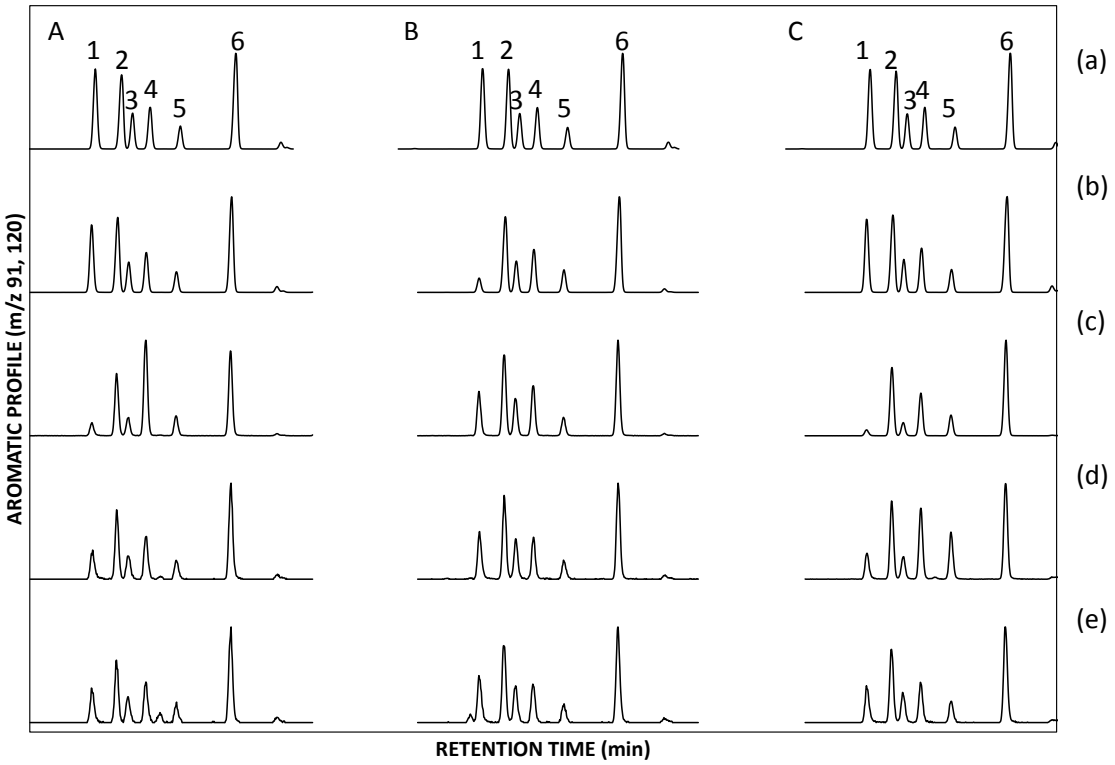


Figure 5-2 Aromatic profile for the soil type comparison of microbial degradation of gasoline on: (A) agricultural soil, (B) residential soil, and (C) industrial soil over (a) 0, (b) 2, (c) 7, (d) 15, and (e) 30 days (Fall sampling). Peaks: (1) propylbenzene, (2) 3-ethyltoluene, (3) 4-ethyltoluene, (4) 1,3,5-trimethylbenzene, (5) 2-ethyltoluene, and (6) 1,2,4-trimethylbenzene.

These trends may be the result of the higher levels of nutrients in the residential and agricultural soils -- both soils contained higher NO_3, NH_4 and K concentrations compared with the brownfield soil (Table 5-3). Furthermore, the residential soil contained more than twice the extractable P compared with the brownfield soil (154 versus 74 mg/kg, respectively). A significant difference in TOC, which indicates the available energy source, might explain differences in microbial degradation; however, the TOC was not significantly different for the fall sampling. Another factor may be the Pb concentration in the brownfield soil (497 mg/kg), which may have impaired the activity

of heterotrophic bacteria. A number of researchers have determined a direct relationship between concentrations of soil Pb and microbial activity in soil [84-86]. Shi and co-workers found that soil Pb decreased microbial activities and led to accumulation of soil organic C; furthermore, Pb was found to pose a greater stress to soil microbes than did other heavy metals [87]. Application of Pb at concentrations of >500 mg kg^{-1} caused an immediate and significant decline in microbial biomass [85]. Zeng and co-workers state that soil Pb concentrations >500 mg Pb/kg may be a "critical concentration", causing a significant decline in soil microbial activity [86]. Clay content also plays a role in bacterial growth, by providing support due to the small particle size which are electrically charged, allowing some bacteria to be adsorbed [88]. However, there are other factors that may have a greater impact as the residential soil, which contained sandy-clay sized particles, showed higher bacterial counts and greater degradation than the agricultural and brownfield soils, which had smaller clay particles.

Principal Components Analysis (PCA) was then used to calculate a set of latent variables that would better explain the overall variance in the data set. Figures 5-3 show the factor loadings and the factor scores from PCA of the fall sampling from each soil type. This biplot shows both the projections of the data in the new factor space and the projections of the original variables. By examining the relative locations of the observations and variables over time, we can determine what variables are more important for a given data point.

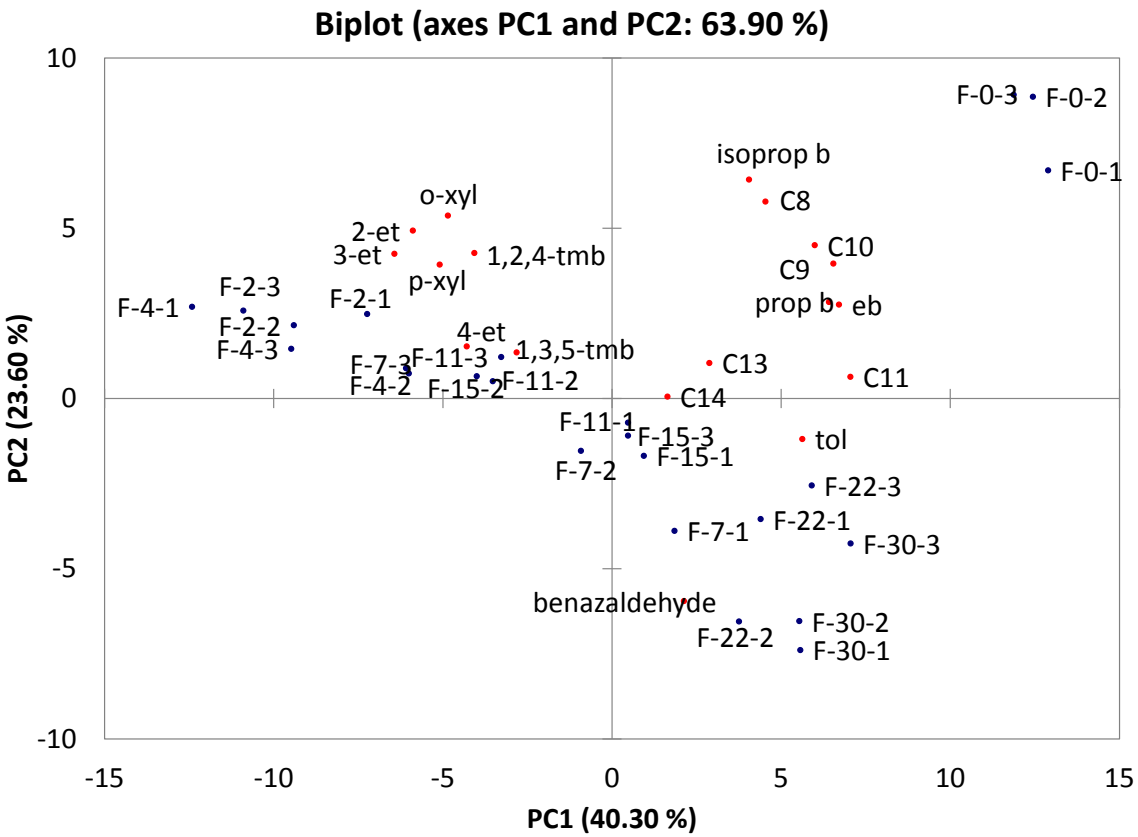


Figure 5-3 PCA biplot showing the factor loadings and the factor scores from the microbial degradation of gasoline on residential soil over 30 days (Fall sampling). Variable abbreviations are as shown in Table 2. Soil samples are designated as “F” for Fall, the number of days of degradation and the replicate number (e.g., F-4-1 is the first replicate from a sample aged four days on Fall soil). Note that the observations begin in the upper right quadrant and progress to the lower right quadrant over the course of 30 days.

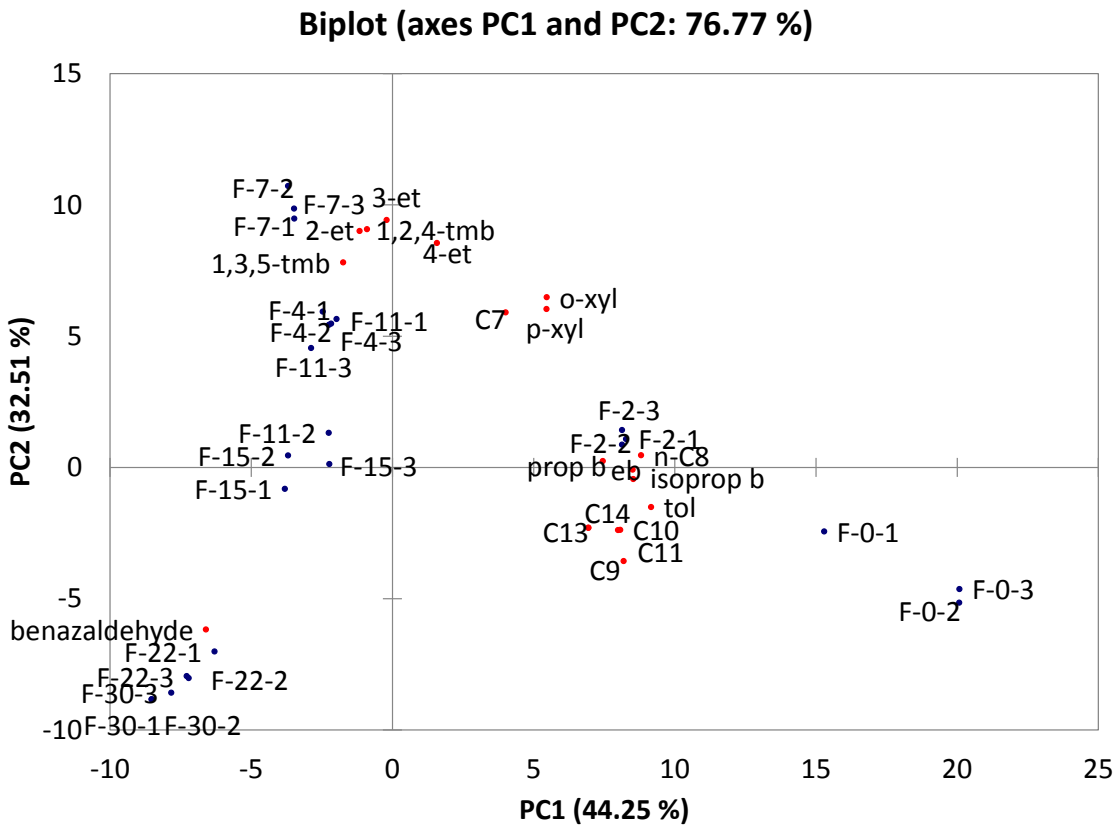


Figure 5-4 PCA biplot showing the factor loadings and the factor scores from the microbial degradation of gasoline on agricultural soil over 30 days (Fall sampling). Variable abbreviations are as shown in Table 2. Soil samples are designated as “F” for Fall, the number of days of degradation and the replicate number (e.g., F-4-1 is the first replicate from a sample aged four days on Fall soil). Note that the observations begin in the lower right quadrant and progress to the lower left quadrant over the course of 30 days.

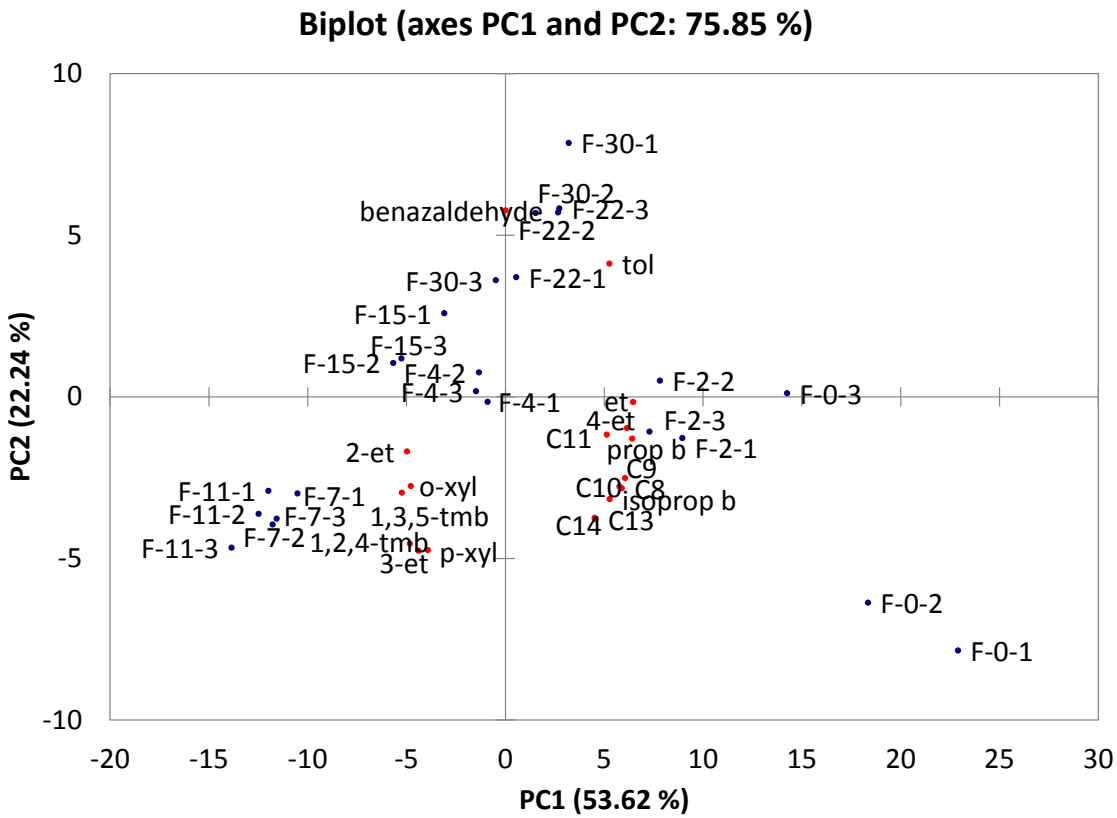


Figure 5-5 PCA biplot showing the factor loadings and the factor scores from the microbial degradation of gasoline on brownfield soil over 30 days (Fall sampling). Variable abbreviations are as shown in Table 2. Soil samples are designated as "F" for Fall, the number of days of degradation and the replicate number (e.g., F-4-1 is the first replicate from a sample aged four days on Fall soil). Note that the observations begin in the lower right quadrant and progress to the upper right quadrant over the course of 30 days.

For example, in the residential soil at day 0, the major contributors to the relative composition of the gasoline samples are the normal alkanes and the mono-substituted alkylbenzenes (Figure 5-3). As degradation proceeds, however, the major contributors to the relative composition of the gasoline samples are the xylenes, ethyltoluenes, and trimethylbenzenes. By days 22 and 30, all of these compounds are completely degraded

and the only compound strongly associated with the gasoline samples is benzaldehyde, which is a suspected degradation product of toluene [89].

The overall degradation rate appears to be slower in the agricultural soil (Figure5-4) as the n-alkanes and the mono-substituted alkylbenzenes are still major contributors in the day 2 samples. Recall that in the residential soil the major contributors in the day 2 samples were the xylenes, ethyltoluenes, and trimethylbenzenes. However, in the end the agricultural soil samples are still significantly degraded by 22 days when the gasoline samples are only associated with benzaldehyde.

The brownfield soil (Figure 5-5) follows a similar trend to the agricultural soil except the day 22 and 30 samples are largely associated with toluene as well as benzaldehyde. This indicates that the brownfield soil was less active than the other soil samples, as toluene is the most abundant compound in gasoline and is one of the first compounds to be significantly decomposed by soil bacteria.

5.3.3.2 Seasonal Comparison

In addition to comparing degradation among different types of soil, a comparison was made between the four seasons for each soil type. However, only a seasonal comparison for the residential soil will be discussed here as it showed the overall greatest degradation. The data for the seasonal comparison for agricultural and brownfield soils can be found in Appendix C.

The alkane profile of gasoline that has been subjected to microbial degradation over the course of 30 days in residential soil over all four seasons is shown in Figure 5-6. Significant losses of the n-alkanes occurred by two days, particularly in all seasons but

summer. It is possible that the summer residential soil was fertilized, providing additional nutrients that bacteria could utilize. It is hypothesized that this would diminish the need to metabolize hydrocarbons for energy and thus slow down the rate of degradation. The bacteria appears to have degraded the aliphatic hydrocarbons in gasoline slightly faster in the winter sampling, although all seasons suffered complete degradation of the aliphatic hydrocarbons present in gasoline by 30 days, leaving behind only aldehydes produced by the bacteria in soil.

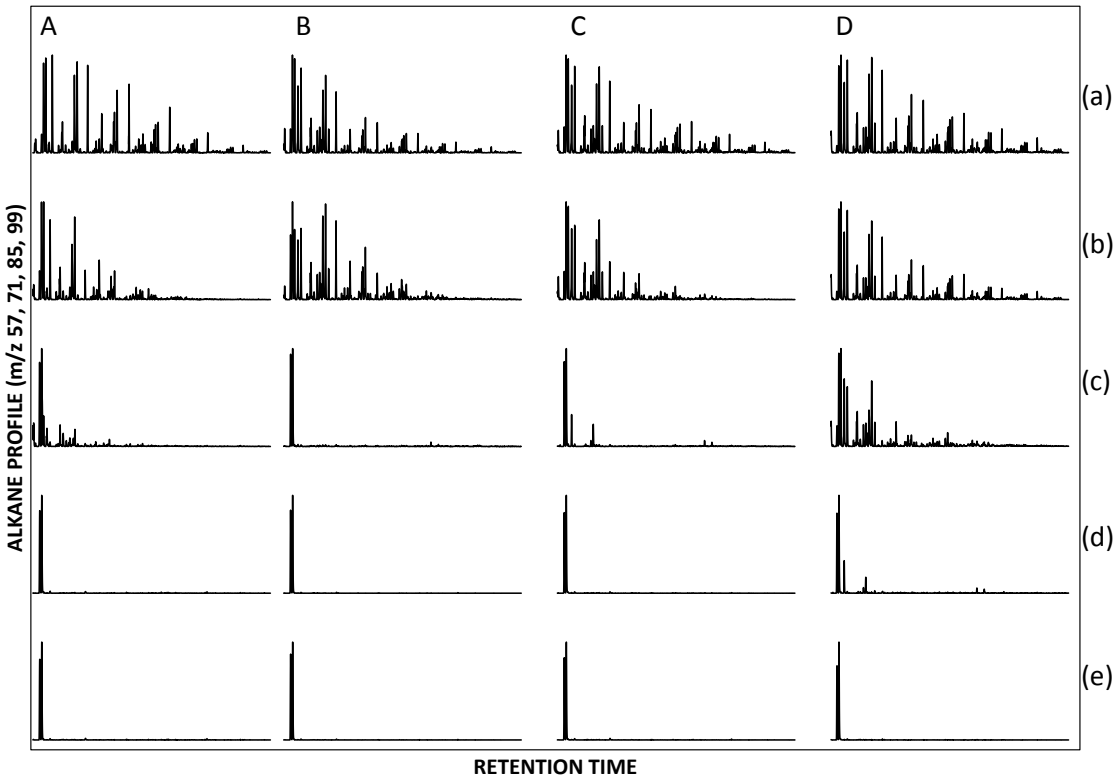


Figure 5-6 Seasonal Comparison of microbial degradation of the alkane profile of gasoline on residential soil in (A) fall, (B) winter, (C) spring, and (D) summer after (a) 0 days, (b) 2 days, (c) 7 days, (d) 15 days and (e) 30 days.

The C_3-alkylbenzenes of the aromatic profile of gasoline subjected to microbial degradation over the course of 30 days in residential soil over all four seasons is shown in Figure 5-7. Bacteria responsible for the degradation of aromatic hydrocarbons significantly reduce the C_3-alkylbenzenes, which are required for the identification of gasoline in a fire debris sample, by 15 days. However, not only must these compounds be present, they must be present in ratios similar to that of a gasoline standard. After just 2 days in three of the four seasons shown in Figure 5-7, the ratios of the C_3-alkylbenzenes may be altered enough that a fire debris chemist would not identify these samples as gasoline. By 30 days all four seasons of the residential soil show significant degradation which would not lead fire debris analysts to identify these samples as gasoline. Furthermore, the fall sampling suffered the greatest loss of the C_3-alkylbenzenes, while the winter sampling seemed to have suffered the least degradation among the C_3-alkylbenzenes.

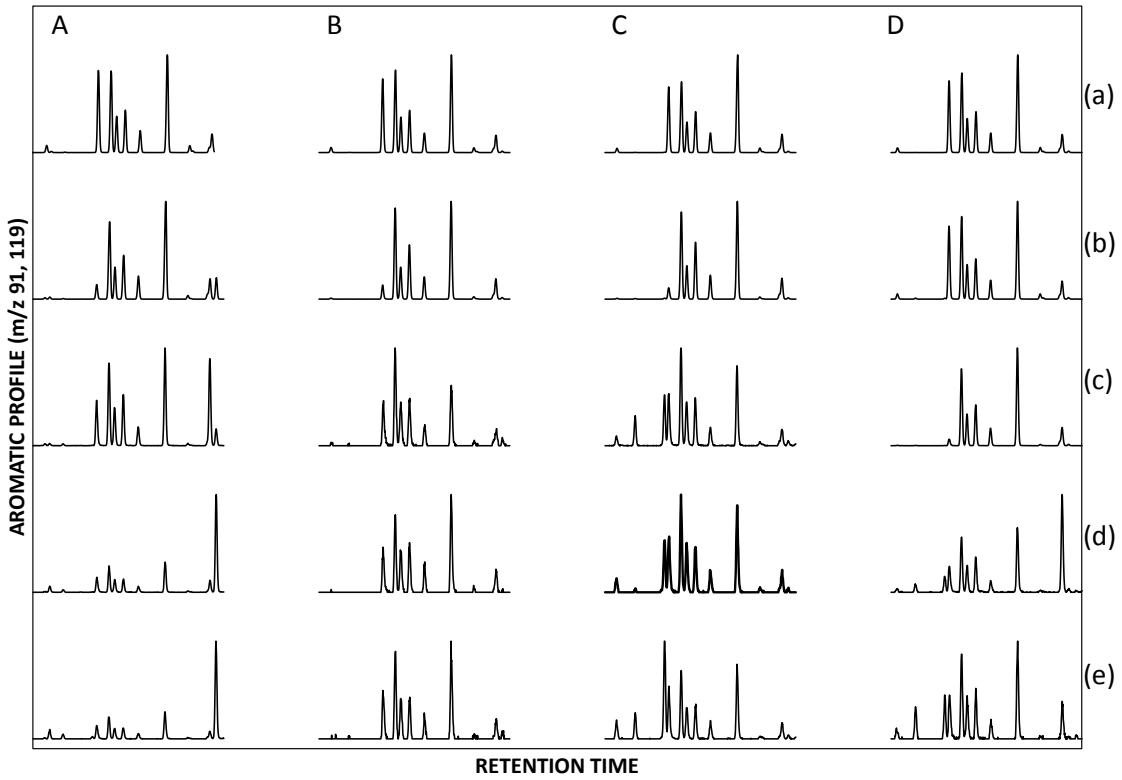


Figure 5-7 Seasonal Comparison of microbial degradation of the C_3-alkylbenzenes of gasoline on residential soil in (A) fall, (B) winter, (C) spring, and (D) summer after (a) 0 days, (b) 2 days, (c) 7 days, (d) 15 days and (e) 30 days.

Principal Component Analysis was also applied to the peak areas of interest for each of the seasons of the residential soil. The biplots showing the factor loadings and the factor scores for each season are shown in Figures 5-8 thru 5-11. Figure 5-8 shows the biplot for the fall sampling of the residential soil. The day 0 samples are projected in the upper right quadrant along with normal alkanes from C_8 to C_{14} and ethyl-, propyl-, and isopropylbenzene. As the samples become more degraded, the samples move away from these compounds. Between 2 and 11 days, the xylenes, ethyltoluenes, and trimethylbenzenes are more highly correlated to the gasoline samples. By 30 days, the

samples are not correlated to any compounds found in gasoline, but are correlated with benzaldehyde. Benzaldehyde is a compound thought to be a byproduct of metabolism.

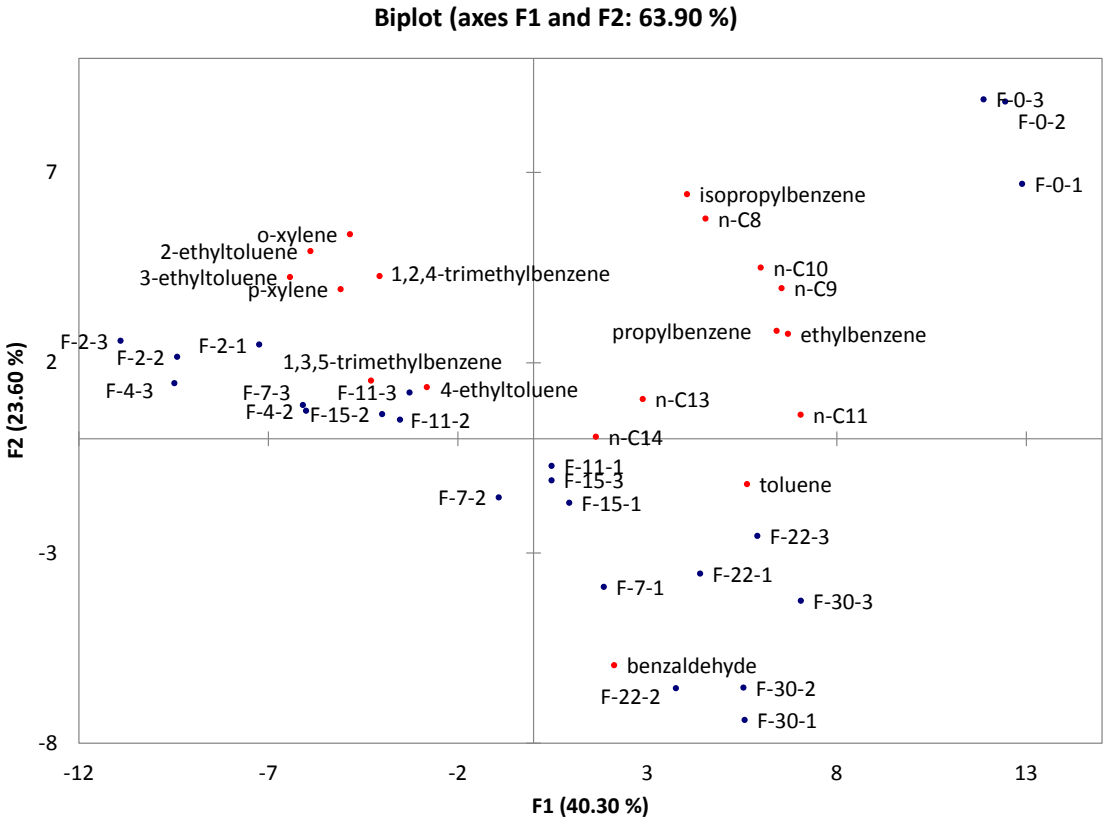


Figure 5-8 PCA biplot showing the factor loadings and the factor scores from the microbial degradation of gasoline on residential soil over 30 for the Fall sampling. Variable abbreviations are as shown in Table 2. Soil samples are designated as "F" for Fall, the number of days of degradation and the replicate number (e.g., F-4-1 is the first replicate from a sample aged four days on Fall soil). Note that the observations begin in the upper right quadrant and progress to the lower right quadrant over the course of 30 days.

Figure 5-9 shows the biplot for the winter sampling of residential soil. The day 0 samples are projected into the upper left quadrant along with toluene, ethyl- and propylbenzene as well as normal alkanes C_{14} and C_{15}. As the samples become more degraded they move away from these lower substituted alkylbenzenes toward the higher substituted ones, such as the trimethylbenzenes. The majority of the normal alkanes are

highly correlated with the day 4 samples but not day 7, suggesting that these compounds are significantly degraded by 7 days. Day 11 through Day 30 samples are projected into the lower left quadrant with benzaldehyde, suggesting that all relevant compounds in gasoline are significantly degraded by 30 days.

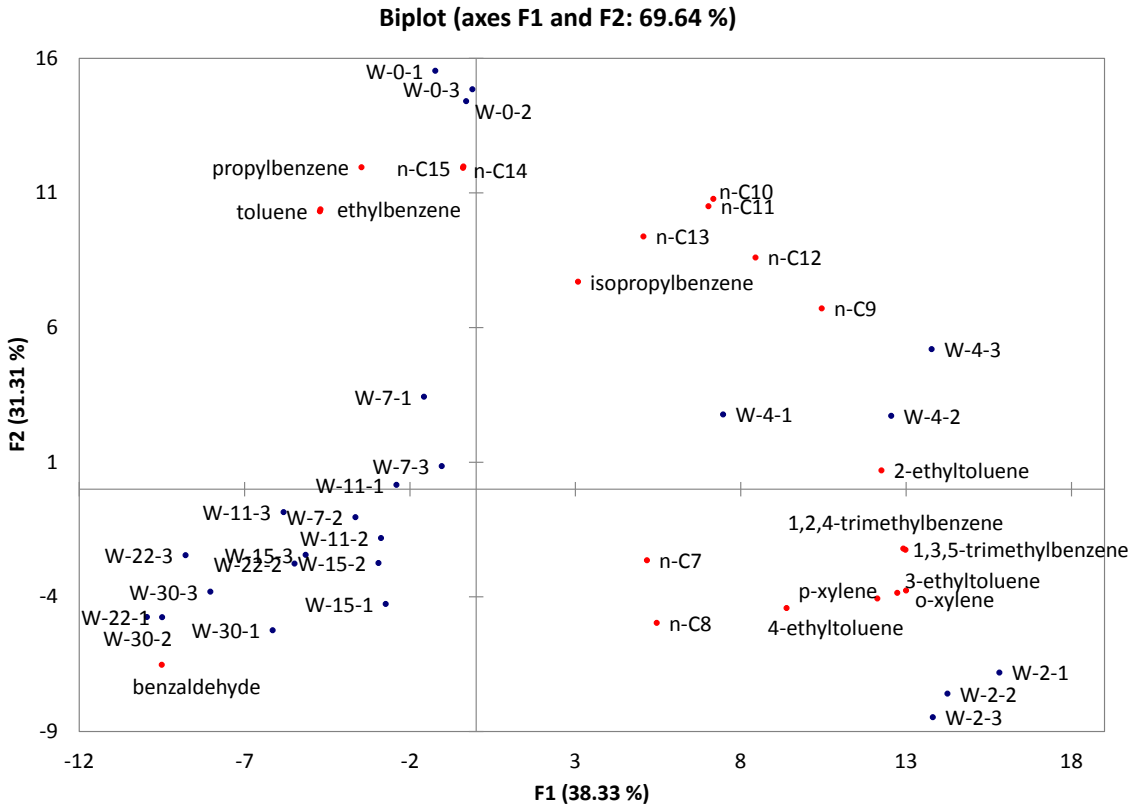


Figure 5-9 PCA biplot showing the factor loadings and the factor scores from the microbial degradation of gasoline on residential soil over 30 for the Winter sampling. Variable abbreviations are as shown in Table 2. Soil samples are designated as "W" for Winter, the number of days of degradation and the replicate number (e.g., W-4-1 is the first replicate from a sample aged four days on Winter soil). Note that the observations begin in the upper left quadrant and progress to the lower left quadrant over the course of 30 days.

The biplot containing the factor loadings and the factor scores for the spring sampling of residential soil is shown in Figure 5-10. Day 0 samples are projected in the upper right quadrant along with the majority of the normal alkanes and toluene. As the

degradation increases, the samples move away from the normal alkanes and become more strongly correlated to the aromatic hydrocarbons. However, after 22 to 30 days, the aromatic compounds are lost as well and the samples become strongly correlated with benzaldehyde and also show some correlation to octane.

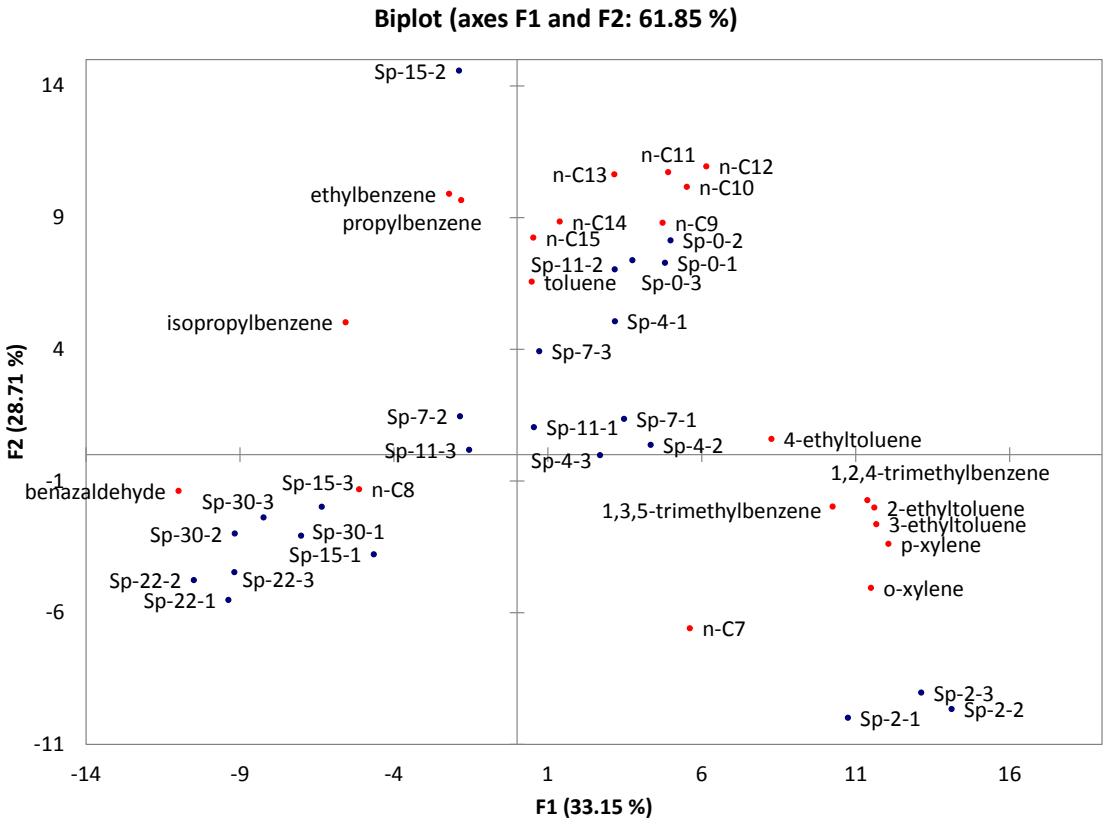


Figure 5-10 PCA biplot showing the factor loadings and the factor scores from the microbial degradation of gasoline on residential soil over 30 for the Spring sampling. Variable abbreviations are as shown in Table 2. Soil samples are designated as “Sp” for Spring, the number of days of degradation and the replicate number (e.g., Sp-4-1 is the first replicate from a sample aged four days on Spring soil). Note that the observations begin in the upper right quadrant and progress to the lower left quadrant over the course of 30 days.

The biplot containing the factor scores and factor loadings for the summer sampling of residential soil is shown in Figure 5-11. Day 0 samples are projected into the upper left quadrant along with ethyl-, propyl-, and isopropylbenzene as well as normal

alkanes C_7 through C_{11}. Toluene and the Day 2 and 4 samples are projected in close proximity to the Day 0 samples and the compounds just mentioned. As degradation progresses, the samples move away from these lesser substituted alkylbenzenes and lower boiling normal alkanes and move toward the higher boiling normal alkanes and higher substituted alkylbenzenes. The summer sampling shows that benzaldehyde is not the only compound correlated to the Day 22 and 30 samples. The strong correlation to these alkylbenzenes suggests that the summer sampling was not significantly affected by microbial degradation.

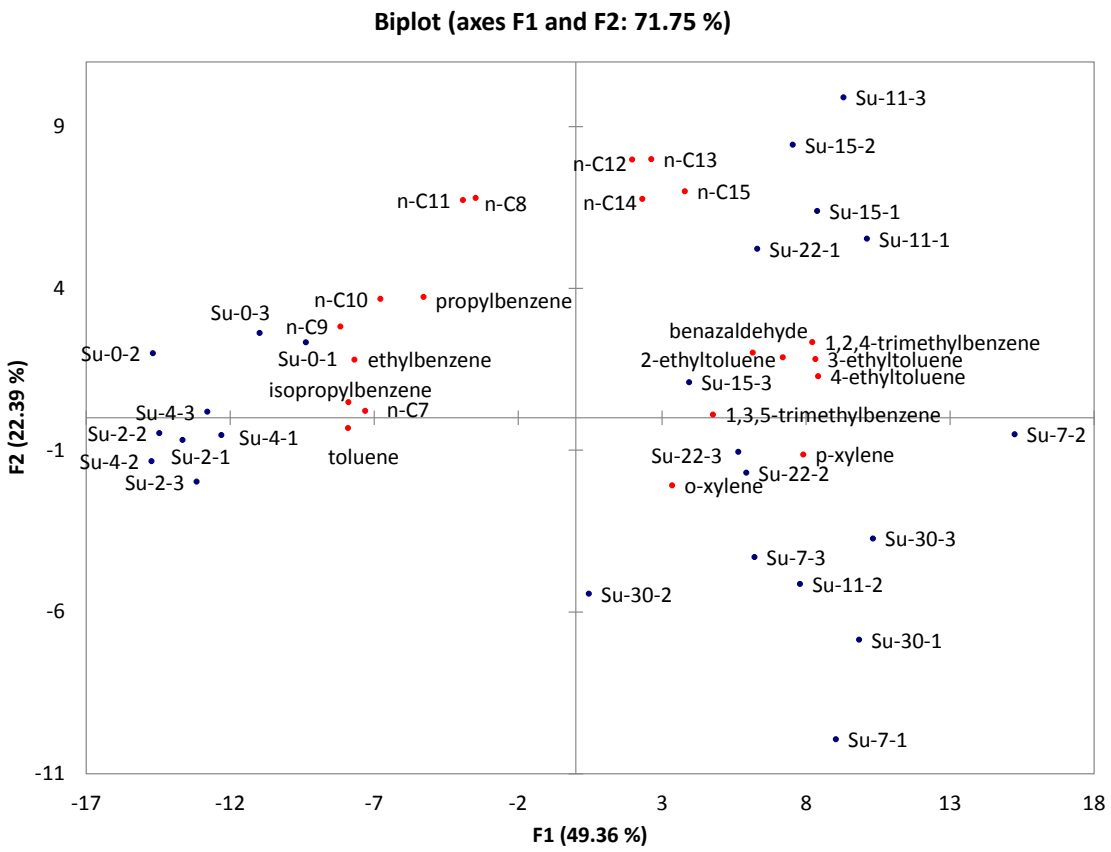


Figure 5-11 PCA biplot showing the factor loadings and the factor scores from the microbial degradation of gasoline on residential soil over 30 for the Summer sampling. Variable abbreviations are as shown in Table 2. Soil samples are designated as "Su" for Summer, the number of days of degradation and the replicate number (e.g., Su-4-1 is the first replicate from a sample aged four days on Summer soil). Note that the observations begin in the upper left quadrant and progress to the lower right quadrant over the course of 30 days.

5.4 Conclusions

For the soil type comparison, microbial degradation was apparent in all soil samples; however, gasoline on brownfield soil suffered the least while gasoline on the residential soil suffered the most and only slightly more than on the agricultural soil. It was expected that the residential soil would suffer the most degradation as the bacteria counts were highest in this soil type. It was also expected that the brownfield soil would show the least microbial degradation due to the increased levels of heavy metals, such as

lead, which is considered toxic to the soil bacteria. Further studies should be conducted to determine how differences in soil toxicity as well as soil nutrients affect bacterial growth. *Pseudomonas* spp., widely recognized as a universal soil-borne microbe capable of degrading many chemical adulterants was not a major metabolic participant in this study, although future work that specifically detects activity of genes encoding known factors for biodegradation would reveal the potential for this genus in these soils.

For the seasonal comparison, the fall sampling showed the most degradation followed by the winter, spring, and summer sampling, which showed the least amount of degradation overall. The summer sampling was initially expected to show the most significant degradation since summer would provide an optimal temperature compared to the other seasons. It is possible that the soil, particularly for the residential and agricultural soils, was fertilized by the caretakers in order to prepare for planting flowers or crops. However, this has not yet been verified by comparison to the soil chemistry data.

CHAPTER 6. PREVENTING DEGRADATION

6.1 Introduction

Fire debris analysis is an important part of a fire investigation when the fire is suspected to have been intentionally set. In these cases, samples can be collected near the suspected point of origin and sent to a suitable laboratory for analysis. At this point, the job of a fire debris chemist is to determine if there is an ignitable liquid residue (ILR) present and if so, to what class the ILR belongs. This paper is concerned with the phenomenon whereby ignitable liquids that absorb into soil at fire scenes are rapidly and irreversibly degraded by microbes. This degradation begins as soon as the ignitable liquid contacts the soil and continues unabated during the often extended period of time between gathering a sample and analyzing it in a forensic science laboratory. Ultimately, the destruction of the ignitable liquid residue can lead to false negative results and negatively impact a fire investigation.

Microbial degradation of ignitable liquids was first reported by Mann [30] and Kirkbride [12], further demonstrated by Chalmers [33], and studied extensively by Turner and Goodpaster [32, 54, 55, 90]. Most recently, it has been reinforced by Hutches [91] that the phenomenon of microbial degradation is not limited to soil as ignitable liquids also degraded on moldy building materials. In general, bacteria in the soil preferentially degrade n-alkanes in the range of C_9 to C_{16} as well as lesser substituted

alkylbenzenessuch as toluene, ethylbenzene and 3-ethyltoluene [32]. In particular, Kirkbride's work identified the species *Pseudomonas putida* in samples exhibiting degradation of aromatic compounds and the species *Pseudomonas fluorescens* in samples exhibiting degradation of aliphatic compounds [12]. Given that bacterial populations can vary depending on soil type and chemistry, it is possible that species of bacteria not identified by Kirkbride could also degrade ignitable liquids. Therefore, it is the aim of this work to identify a method that can kill nearly all bacteria in the soil in order to ensure the preservation of fire debris samples.

Classical sterilization methods include moist heat sterilization (autoclaving), dry heat sterilization, γ-irradiation, microwaves, gaseous chemicals, and the addition of chemical solutions such as mercuric chloride and sodium azide [22-28]. Many of these and other methods reported in the literature are either not ideal for use in the field or they do not eliminate all living bacteria in the soil [22-29, 92]. A chemical treatment would be a more efficient means for treatment of field samples. An ideal antimicrobial solution for the use in fire debris samples should be water soluble, non-volatile (e.g., a polar organic compound with a high molecular weight), relatively non-toxic to humans, does not interfere with sample analysis, and is easy to use by non-scientists, who will be the primary users of the solution. Overall, a chemical treatment that could be deployed immediately upon collecting a sample would be a more efficient means for treatment of fire debris. The nearest approximation of such a procedure would be the preservation of volatile organic compounds in environmental soil samples – which can be accomplished by forming an acidic slurry using high concentrations of salts like sodium chloride and sodium bisulfate [93]. However, and as will be discussed, this approach was found to be

less effective than other options for fire debris. Triclosan is a known antimicrobial used in hand soaps, lotions and other household products. Ultimately, our results will show that triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) can act as a highly effective preservative for fire debris.

6.2 Materials and Methods

6.2.1 Materials

Triclosan (2,4,4'-Trichloro-2'-hydroxydiphenyl Ether) was purchased from TCI America. Sodium hydroxide pellets were purchased from Fisher Scientific. Quart-size paint cans were purchased from Best Containers. Charcoal strips were purchased from Albrayco Technologies. Gasoline (97 octane) was purchased from a local gas station. Household products (bleach, hydrogen peroxide, vinegar, Betadine (10% povidone-iodine) and Hibiclens (4.0% w/v chlorhexidine gluconate)) were purchased from Walmart. Sodium azide and methanol were purchased from Sigma-Aldrich. Tryptic Soy Broth, sodium chloride, sodium hydroxide copper sulfate, glycerin, inoculating loops, screw-capped culture tubes, petri dishes, conical shaped plastic culture tubes, and plastic cuvettes were purchased from Fisher Scientific. The following ingredients to make minimal media were also purchased from Fisher Scientific: magnesium sulfate heptahydrate, citric acid monohydrate, potassium phosphate dibasic anhydrous, and sodium ammonium phosphate tetrahydrate. Minimal media is a growth medium that contains few salts and a single carbon source which only supports bacteria that can synthesize their own amino acids.

Tryptic Soy Broth was prepared as per the package instructions. Concentrated minimal media was prepared with approximately 2.5g magnesium sulfate heptahydrate

($MgSO_4 \cdot 7H_2O$), 27.3g citric acid, 125g potassium phosphate dibasic (K_2HPO_4), and 43.7g sodium ammonium phosphate ($NaNH_4HPO_4$) in 330mL deionized (DI) water. 20mL of the concentrated minimal media solution was diluted in 480mL DI water and sterilized in an autoclave for 30 minutes. Tryptic Soy Agar (TSA) plates were prepared by dissolving 20g of the medium in 500mL DI water with heat and continuous stirring. The media was then autoclaved for 15 minutes and poured while still hot into sterile plastic Petri dishes and covered. Once solidified, a sterile 10µL loop was used to streak the plates. These agar plates were allowed to incubate at room temperature overnight.

6.2.2 Methods

6.2.2.1 Classification of Bacterial Species

For bacterial species classification, a soil extract was prepared from approximately 6.3 g of potting soil in 50mL sterile saline (0.9% w/v). 100mL of Tryptic Soy Broth (TSB) was prepared per package instructions, sterilized, inoculated with 10µL of the soil extract and incubated at room temperature for 48h. This culture served as the source of soil bacteria for all subsequent experiments.

A 0.1% v/v mixture of gasoline in sterile minimal media was inoculated with soil bacteria and incubated at room temperature with shaking for 5 days, whereupon bacterial growth was evident. Both positive and negative controls were also included to ensure the viability of the soil bacteria and sterility of the materials. Sub-cultures were then prepared in both minimal media and TSB, incubating at room temperature with shaking for 48h. Bacterial growth was consistently seen in the positive controls as well as in inoculated cultures containing 0.1% v/v gasoline.

The final sub-culture was streaked onto a TSA plate and a single colony of the bacteria was transferred to a clean glass slide. The bacteria were spread out with a few drops of DI water and the cells were heat fixed to the slide by gently passing the slide thru the flame of a Bunsen burner until the water evaporated. A few drops of crystal violet dye were applied for approximately 1 minute. The slide was then rinsed gently with DI water to remove excess dye. This procedure was repeated with iodine followed by an alcohol/acetone rinse. Finally, a few drops of safranin were applied for approximately 45 seconds and then the excess stain was rinsed off with DI water.

Upon determining the morphology and possibly what type of bacteria was isolated, a culture was prepared on *Pseudomonas F* agar slant and allowed to grow overnight. This media allows for the differentiation of Pseudomonas from other types of bacteria, as the media fluoresces under UV light in the presence of Pseudomonas. Then the culture was held under a UV light to determine if the bacteria were fluorescent.

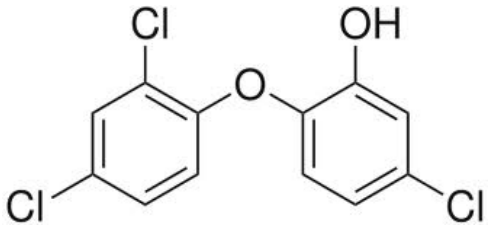
6.2.2.2 Sterilization of Bacterial Cultures

Tryptic Soy Broth was prepared per package instructions. 10mL of the media was transferred to screw-capped culture tubes and then autoclaved with caps loose to ensure sterility. Bacteria were transferred to the media using a 10µL disposable loop from a 5g soil sample mixed with 2mL of 0.9% sterile sodium chloride solution. The cultures were allowed to incubate overnight at room temperature on a standard analog shaker table (VWR) to allow for bacterial growth. Various solutions of chemical agents (including several household products) were then added volumetrically to the growth media. The

treated cultures were then incubated again overnight, sub-cultured into fresh media using a 10µL loop, and then monitored for growth.

6.2.2.3 Sterilization of Soil

Soil samples were also treated directly by adding 2mL of test solutions in various concentrations to 5g of soil and mixed well. The samples were then allowed to sit overnight and then sub-cultured using the same procedure as above. All chemicals were diluted in water except for triclosan (Figure 1), which has poor water solubility. It was discovered later that the solubility of triclosan is increased significantly at high pH, so triclosan was ultimately dissolved in a dilute solution of sodium hydroxide.



Physical Properties	
Molecular Weight	289.54g/mol
Water Solubility	17mg/L
Melting Range	131-134.6°F

Figure 6-1 Structure and relevant physical properties of triclosan.

6.2.2.4 Growth Study

The ability of triclosan to sterilize bacterial cultures was monitored over time using UV/visible absorbance spectroscopy. Soil samples were treated with 1.8% triclosan in 0.1M NaOH (5mL per 2g of soil). Control samples were also prepared using water and

0.1M NaOH. All samples were prepared in triplicate and gently shaken for 60s at room temperature on a shaker table. 10µL of the supernatant was then transferred to 10mL of TSB. The absorbance of the solutions were monitored for up to 77 hours via an HP single beam diode array UV-Vis Spectrophotometer in the 400 to 800nm range. Absorbance was recorded at 600nm.

6.2.2.5 Laboratory Studies Using Passive Headspace Analysis

Approximately 100g of soil was placed into a quart-sized paint can and spiked with 20µL of gasoline. Then 60mL of either bleach or triclosan was added and mixed well to ensure that the soil was completely saturated. Controls were also prepared using water and 0.2M sodium hydroxide in addition to samples with no additional treatment (*i.e.*, only soil and ignitable liquid). The samples were aged for 0, 2, 7, 11, 15, 22, and 30 days. After the specified time period, a third of a charcoal strip was suspended into the headspace of the paint can on a pre-baked paper clip using a nylon string. The cans were baked at 85°C for 4h. Upon cooling, the charcoal strips were extracted with 400µL of pentane. The samples were then analyzed using an Agilent 6890N GC containing a DB-5 30m x 0.25mm x 0.25micron column and an Agilent 5975 MSD. The method utilized is a standard method for the analysis of fire debris and consists of a 250°C inlet temperature, an injection volume of 1µL, an initial oven temperature of 40°C held for 3 minutes, temperature gradient of 10°C/min up to a final temperature of 280°C, held for 3 minutes. A 2 minute solvent delay was used along with a scan range of 40-300m/z.

6.2.2.6 Field Studies Using Passive Headspace Analysis

Several areas of approximately 3’ by 3’ each were dug up in a grass lawn to remove the grassy layer and expose the underlying soil. Soil control samples were collected prior to exposure to the ignitable liquid from the Molotov Cocktails. For each site, a Molotov cocktail was constructed by the Indianapolis Fire Department using a beer bottle filled to the neck with gasoline and a cloth wick. With additional firefighters and fire suppression equipment on standby, a firefighter in turn-out gear then threw the device against a brick in the center of the patch of soil, thereby igniting a small blaze. After the fire self-extinguished, glass bottle fragments were collected and then soil from the sites was collected and homogenized before separating into 24 quart-sized paint cans for analysis. Half of the cans received approximately 100mL of 2% triclosan in 0.2M sodium hydroxide, which was enough to completely cover the soil. Half of the control samples were also treated with this triclosan solution. The cans were then sealed and allowed to age for up to 154 days. Samples were analyzed starting on day 0 and every two weeks thereafter. After the specified time period, a third of a charcoal strip was suspended into the headspace of the paint can on a pre-baked paper clip using a nylon string. The cans were baked at 85°C for 4h. Upon cooling, the charcoal strips were extracted with 400µL of 0.01% tetrachloroethylene in pentane.

All samples were analyzed using an Agilent 6890N GC containing a DB-5 30m x 0.25mm x 0.25micron column and an Agilent 5975 MSD. The method utilized is a standard method for the analysis of fire debris and consists of a 250°C inlet temperature, an injection volume of 1µL, an initial oven temperature of 40°C held for 3 minutes,

temperature gradient of 10°C/min up to a final temperature of 280°C, held for 3 minutes. A 2 minute solvent delay was used along with a scan range of 40-300m/z.

6.3 Results and Discussion

6.3.1 Classification of Bacterial Species

The bacteria that were isolated from the culturing study were presumptively identified as *Pseudomonas*, the same genus isolated by Kirkbride [12]. The cultures spiked with 0.1% gasoline were grown in both a general media (TSB) containing all the essential nutrients for growth and in a minimal media where nutrients were fairly limited. In TSB, growth occurred within 24hr; however, growth did not occur in the minimal media until 7 days had passed. These cultures were sub-cultured to isolate single species of bacteria that could degrade gasoline. Again, growth occurred after 24hr in the TSB spiked with gasoline and after 7 days in the minimal media spiked with gasoline. These cultures were then sub-cultured again onto a TSA plate. Growth was observed after 24hr. An isolated colony was determined to contain gram-positive short rods, which describes *Pseudomonas*. A *Pseudomonas F.* agar slant showed, by fluorescence, that the isolated bacteria could be presumptively identified as *Pseudomonas F.*

6.3.2 Sterilization of Bacterial Cultures

Initial sterilization experiments involved treating cultures containing soil bacteria with various chemical preservatives. The treated cultures were then used to inoculate sub-cultures, which were monitored for bacterial growth. The maximum dilution of the solutions that generated a sterile sub-culture is listed for each solution in Table 6-1, ranging from 2% v/v for bleach to 29% v/v for hydrogen peroxide. Table 6-1 also lists the concentration of the active ingredient in the final diluted solution. In this case, the

most potent sterilizing agent was bleach (0.12% w/v) and the least potent sterilizing agent was sodium bisulfate (1.1% w/v). Although it was encouraging that soil bacteria could be effectively eliminated with a variety of chemical agents, each solution had at least one potential drawback if it were to be applied to the preservation of fire debris. As discussed above, an ideal antimicrobial solution for fire debris samples would be water soluble, non-volatile, non-toxic, easy to use, and it would not interfere with subsequent sample analysis for ignitable liquid residues. However, the solutions that were evaluated in this set of experiments suffered from issues such as toxicity, reactivity, volatility and low potency.

Table 6-1 The maximum dilution (and minimum concentration of active ingredient) required for various solutions so that they sterilized a tryptic soy broth (TSB) containing soil bacteria.

Solution	**Maximum Effective Dilution (v/v)**	**Minimum Effective Concentration**	**Potential Drawback**
Bleach (6% w/v sodium hypochlorite)	2.0%	0.12% w/v	Strong Oxidizer
Sodium Azide (6.5% w/v)	3.9%	0.25% w/v	Highly Toxic
Vinegar (5% v/v acetic acid)	16.7%	0.84% v/v	Highly Volatile
Hydrogen Peroxide (3% v/v)	28.6%	0.86% v/v	Strong Oxidizer
Sodium Bisulfate (12% w/v)	9.1%	1.1% w/v	Low Potency

6.3.3 Sterilization of Soil

At this point, testing moved from treating cultures to treating actual soil samples as would be done in the field. This is a more difficult task as the bacteria are entrained within the soil matrix, which itself contains a complex array of inorganic and organic compounds that could interfere with a potential sterilizing agent. For example, when solutions of bleach were used to treat soil, the minimum effective concentration for sterilizing soil was 0.72% w/v, six times higher than what was previously seen with a culture sample. Furthermore, the relatively low concentration of sodium azide used to sterilize cultures was ineffective for sterilizing soils. The toxicity and disposal of this compound also made it unattractive for further study.

Table 6-2 lists a series of non-volatile bacteriocides that were evaluated. Betadine and Chlorhexidine are solutions used in hospitals as disinfectants; however at concentrations of 20% and 14% in water they were ineffective in killing soil bacteria. Copper sulfate has also been known to kill bacteria but even at a concentration of 5%, samples showed bacterial growth after 24 hours on a nutrient rich growth medium. In contrast, the anti-bacterial agent triclosan was tested in a methanol solution and was found to be effective at 1% w/v for 14 days. However, methanol is not an acceptable solvent for this application as it is highly volatile, flammable and potentially encountered in fire debris samples. Given that triclosan is not very water soluble, solvents such as glycerin and glycerin/water were tested as a means to increase solubility. While these solutions did aid in the solubility of triclosan in water, the resulting triclosan solutions were less effective than 1% triclosan in methanol. In all cases, control samples were used to ensure that the solvent itself did not inhibit bacterial growth. The solubility problem

was solved when we tested the solubility of triclosan at high pH and determined that triclosan is readily soluble up to nearly 2% w/v in 0.1M sodium hydroxide. This solution was carried forward into further testing.

Table 6-2 Effectiveness of various antimicrobial solutions for sterilizing soil samples. After treating the soil, a 10 µL sample of the supernatent was used to streak TSA plates, which were monitored for bacterial growth over time.

Solute (Solvent)	Minimum Effective Concentration	Effective Period
Triclosan (Glycerin)	0.50% w/v	7 days
Triclosan (MeOH)	0.50% w/v	7 days
Triclosan (MeOH)	1% w/v	14 days

6.3.4 Spectroscopic Analysis

The growth or lack thereof in cultures prepared from soil samples treated with 1.8% triclosan in 0.1M sodium hydroxide was measured using an absorbance spectrometer. While the control samples of water and 0.1M sodium hydroxide show a general trend of increasing absorbance due to the increased bacterial growth (which increases the turbidity of the solution), the triclosan samples showed no increase over the duration of the experiment (Figure 6-2).

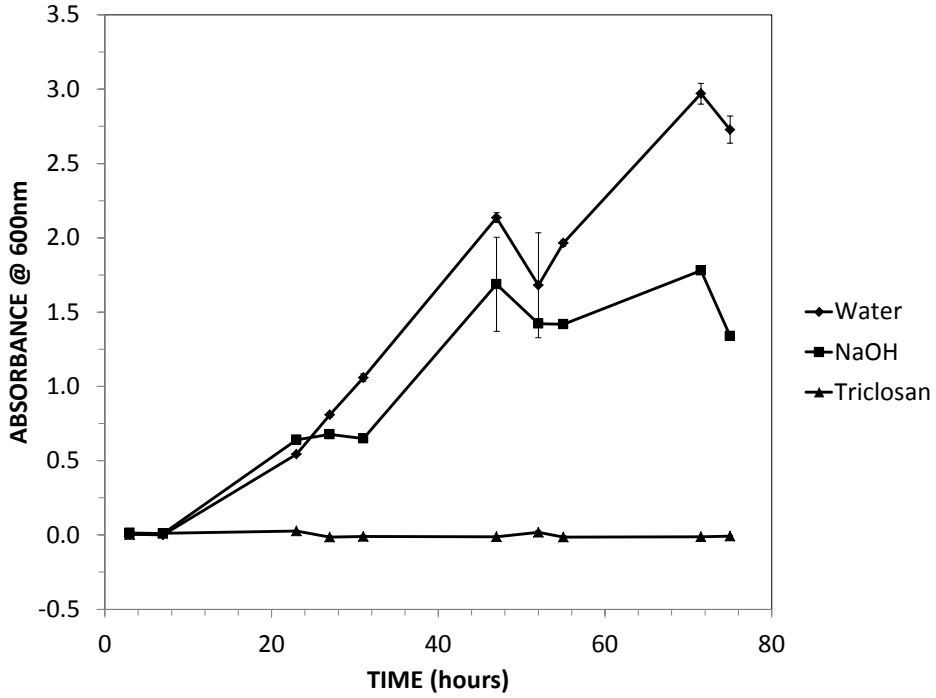


Figure 6-2 A growth curve constructed using UV-Vis analysis of cultures taken from soil exposed to water (control), 0.1M NaOH (control) and 1.8% w/v Triclosan in 0.1M NaOH.

6.3.5 Laboratory Studies Using Passive Headspace Analysis

Passive headspace analysis was performed to compare the effectiveness of triclosan and bleach in soil samples spiked with gasoline over a longer period of time, corresponding to how long an actual fire debris sample may be stored prior to analysis. Soil samples spiked with gasoline were treated with bleach and 2% triclosan in 0.2M sodium hydroxide. Positive control samples were left untreated. Additional control samples were treated with water or 0.2M sodium hydroxide. Figure 6-3 compares the C_3-alkylbenzenes from the total ion chromatograms of degraded gasoline (gasoline left untreated) to gasoline treated with undiluted household bleach and 2% triclosan in 0.2M sodium hydroxide. Gasoline left untreated is significantly altered by the bacteria within days while the gasoline samples treated with triclosan remain unaffected even after one

month. It is interesting to note that while bleach was just as effective as triclosan in the spectroscopic analysis, it was less effective than triclosan in the passive headspace analysis. This might be due to some of the soil not getting exposed to the bleach as the ratio of soil to solution was less in the passive headspace experiments than in the spectroscopic experiments, although the soil was completely covered with the chemical solution. In the case of bleach, if there were clumps of soil where the bleach did not penetrate, some of the bacteria would be shielded and therefore were able to continue metabolizing the selected hydrocarbons in the gasoline. Additionally, bleach oxidized the metal paint cans used to contain the samples, which reduces the available sodium hypochlorite to work against the bacteria.

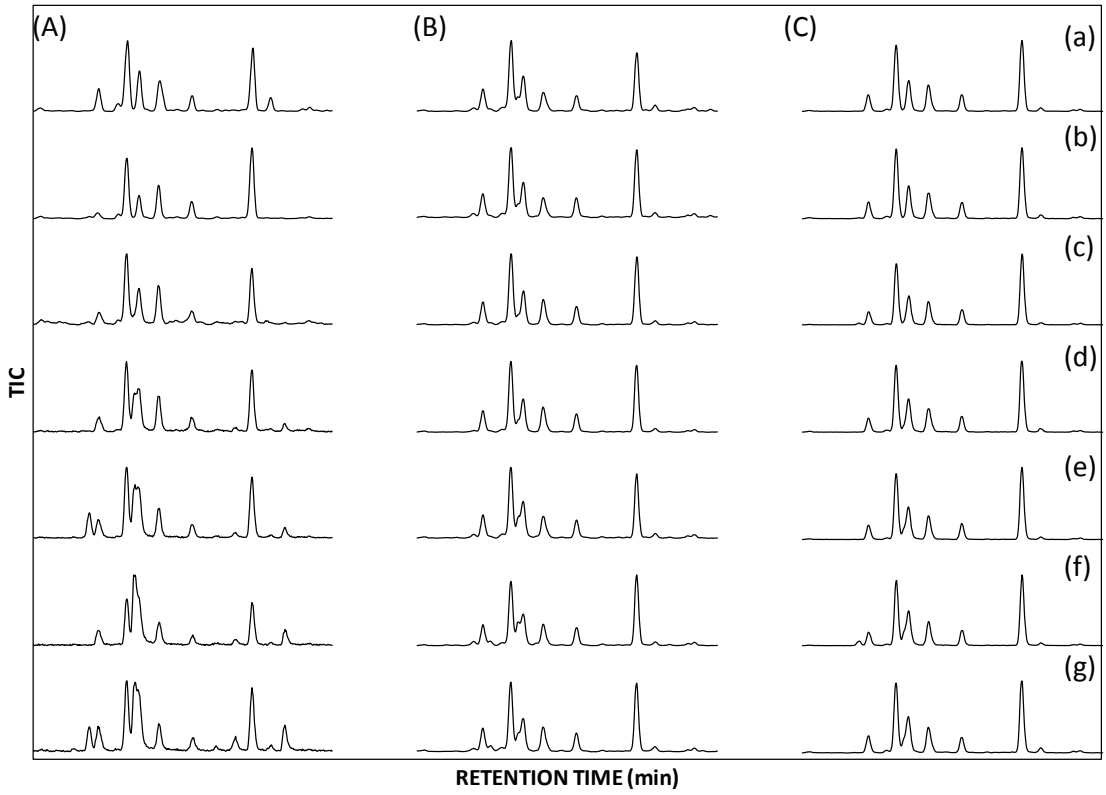


Figure 6-3 The group of 5 in gasoline: degradation (A) versus preservation with (B) bleach and (C) 2% Triclosan in 0.2M NaOH after (a) 0, (b) 2, (c) 7, (d) 11, (e) 15, (f) 22, and (g) 30 days.

Figure 6-4 is a comparison of chromatograms from untreated gasoline to triclosan treated gasoline samples on three different types of soil. This figure shows that all samples left untreated are all significantly degraded after 15 days, with the residues on the industrialized soil suffering the least and the residues on the residential soil suffering the most. The most abundant peak in the top row of chromatograms is the internal standard peak for tetrachloroethylene. Figure 6-4 also shows that all samples treated with triclosan for 15 days were still unaffected. Even after 30 days samples treated with triclosan remained unaltered.

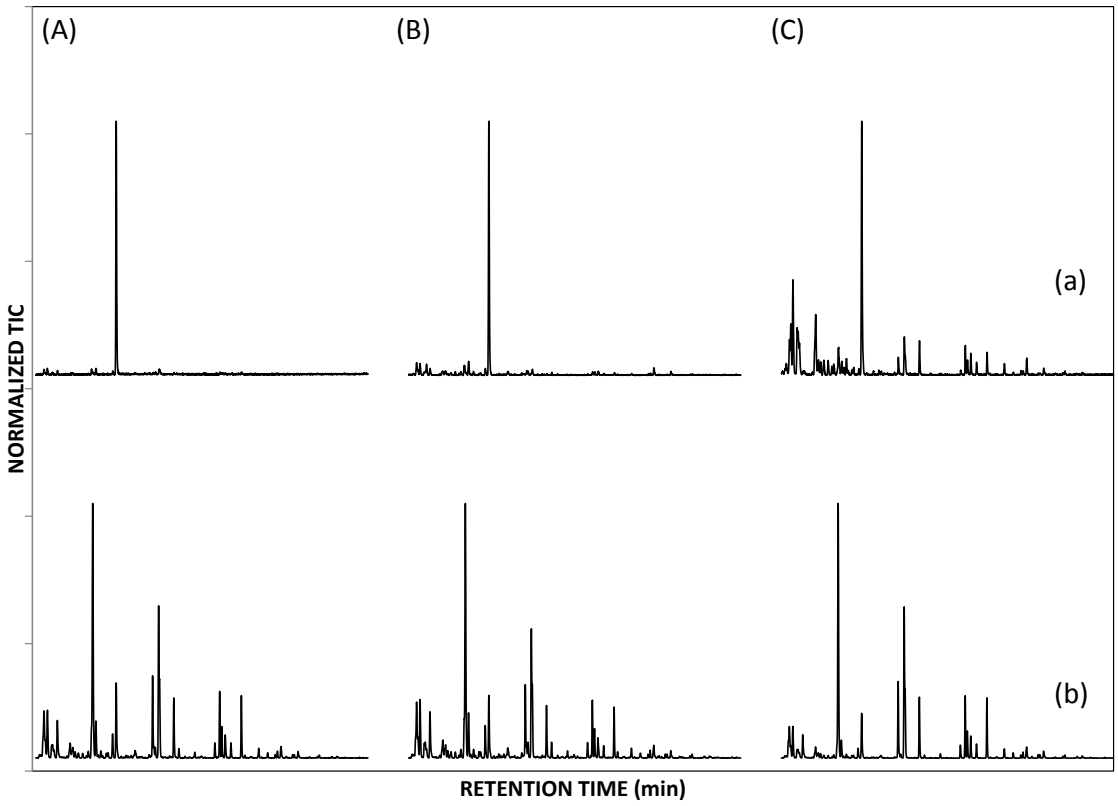


Figure 6-4 Total ion chromatogram comparing degraded gasoline (a) versus preserved gasoline (b) after 15 days on (A) residential soil, (B) agricultural soil, and (C) industrial soil.

6.3.6 Field Studies Using Passive Headspace Analysis

Two studies were conducted, the first in August 2011 and the second in July 2012. The summer 2012 was unseasonably warm and dry, which may explain why little to no degradation was observed in untreated samples. Therefore only data from the first study will be presented and discussed here. The data from the second study can be found in Appendix D. The first study was designed to show the efficacy of triclosan for preserving gasoline in incendiary samples. Figure 6-5 shows the Total Ion Chromatogram (TIC) of gasoline on soil collected from site 1 without treatment (Figure 6-5A) and with treatment using 2% triclosan in 0.2M sodium hydroxide (Figure 6-5B). Overall, treatment with

triclosan proves to be effective for preserving the chromatographic profile of gasoline over 140 days while untreated samples were degraded. For example, Figure 6-5A clearly shows a significant reduction in the relative amount of toluene, the xylenes, and isopropylbenzene. Additionally, the peak height ratio of *m*-& *p*-xylene (peak 4) and *o*-xylene (peak 5) reverse after 28 days.

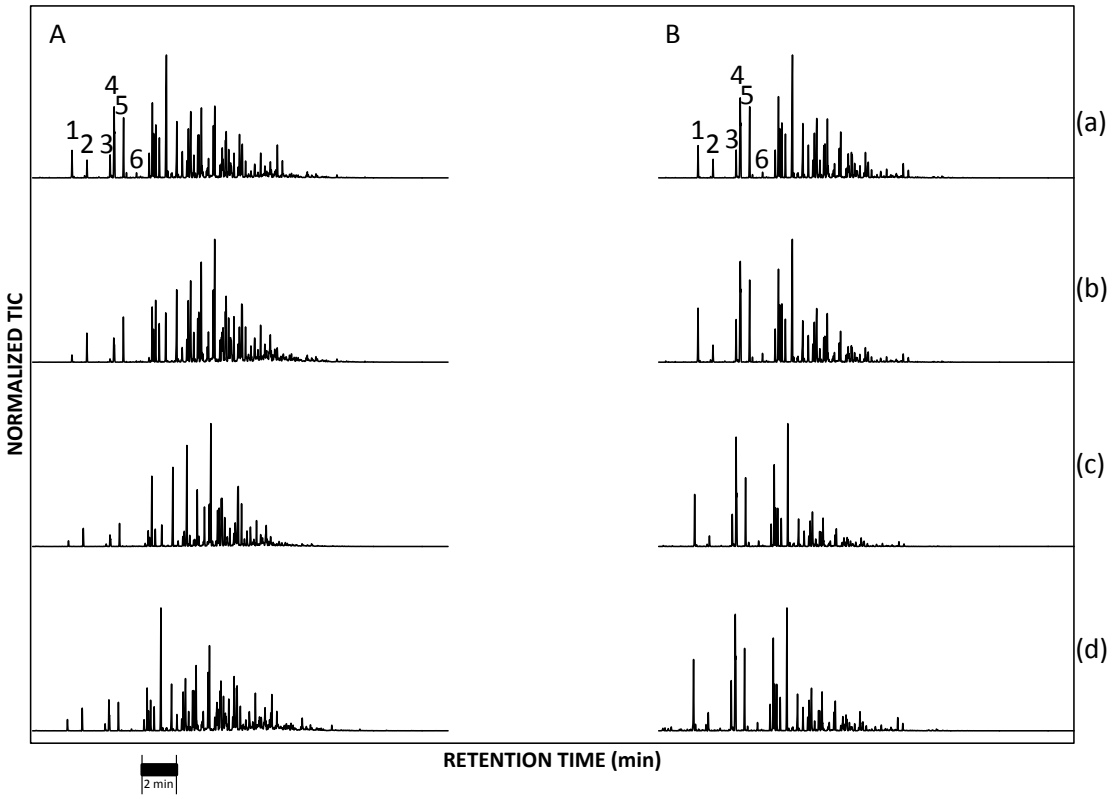


Figure 6-5 TIC of gasoline for site #1 showing (A) microbial degradation versus (B) preservation with 2% triclosan in 0.2M sodium hydroxide in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days. Peaks: (1) toluene, (2) tetrachloroethylene, (3) ethylbenzene, (4) *m*- & *p*-xylene, (5) *o*-xylene, (6) isopropylbenzene.

Figure 6-6 shows the portion of the TIC corresponding to the C_3-alkylbenzenes without treatment (Figure 6-6A) and with treatment (Figure 6-6B) for up to 140 days. The chromatographic profiles of untreated samples exhibited extensive changes in

relative peak heights. For example, propylbenzene, the ethyltoluenes, and 1,3,5-trimethylbenzene are significantly reduced compared to 1,2,4-trimethylbenzene after 140 days. This is important as the C_3-alkylbenzenes are used in the identification of gasoline in a fire debris sample. Not only must the C_3-alkylbenzenes be present in a gasoline sample, but they must be present in ratios similar to that of a gasoline standard.

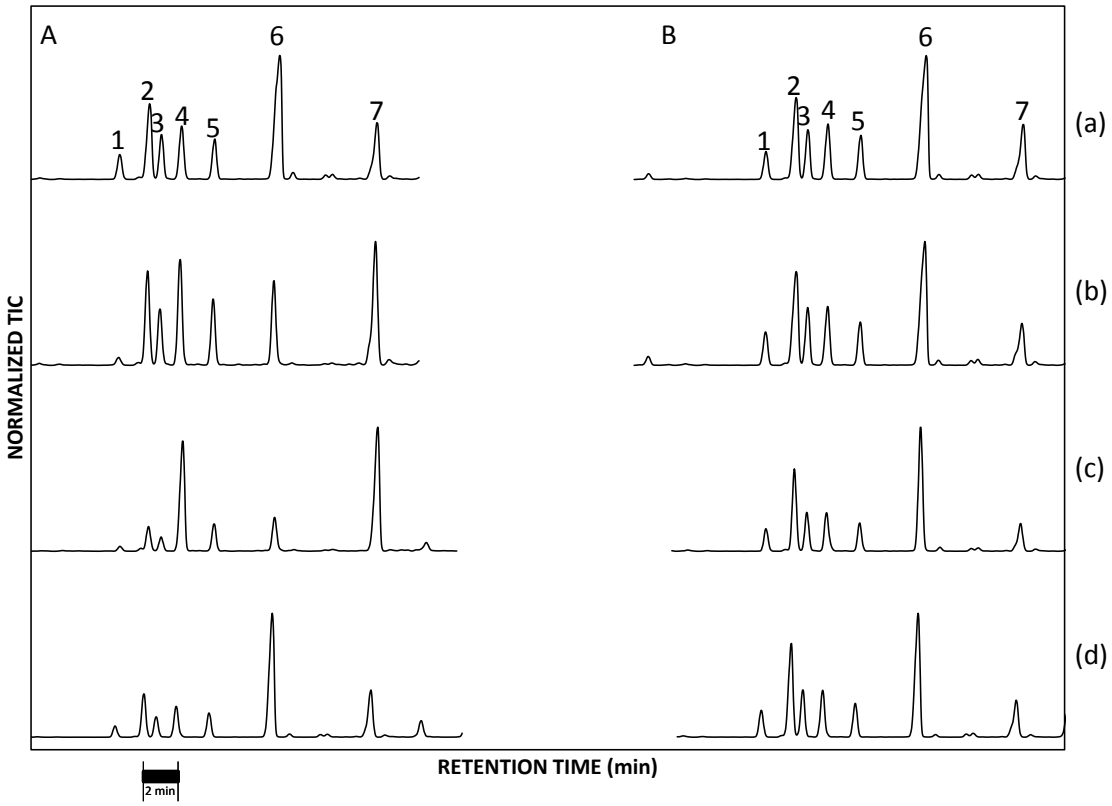


Figure 6-6 C_3-alkylbenzenes in gasoline for site #1 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days. Peaks: (1) propylbenzene, (2) 3-ethyltoluene, (3) 4-ethyltoluene, (4) 1,3,5-trimethylbenzene, (5) 2-ethyltoluene and (6) 1,2,4-trimethylbenzene (peak (7) was also identified as a trimethylbenzene isomer by mass spectral library search).

Figure 6-7 shows the extracted ion profile corresponding to alkanes (i.e., m/z 57, 71, 85, 99) from gasoline on soil collected from site 1 without treatment (Figure 6-7A) and with treatment (Figure 6-7B). In this case, the normal alkanes are significantly

reduced within 28 days in the untreated samples, while they are preserved in the treated samples.

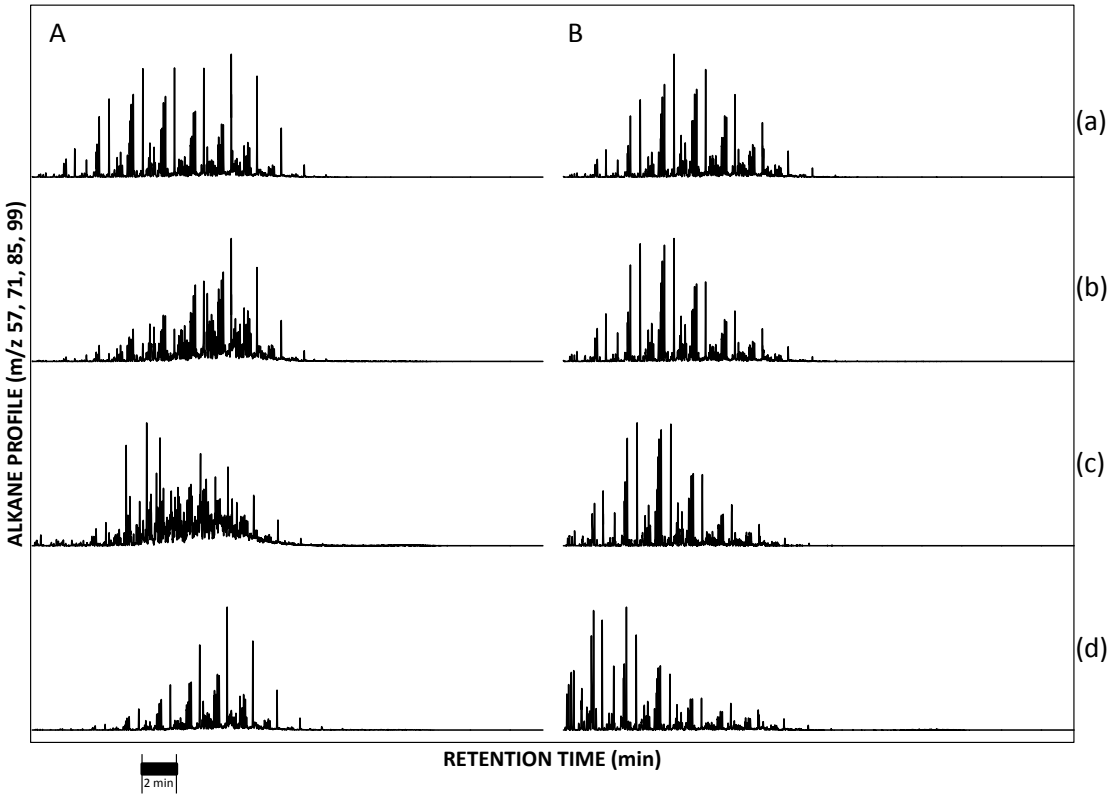


Figure 6-7 Alkane profile of gasoline for site #1 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days.

Figure 6-8 shows the Total Ion Chromatogram (TIC) of gasoline on soil collected from site 2 without treatment (Figure 8-8A) and with treatment using 2% triclosan in 0.2M sodium hydroxide (Figure 6-8B). As in site 1, the TIC reveals significant loss of the lesser substituted alkylbenzenes such as toluene and xylenes compared to the volumetric internal standard, tetrachloroethylene (peak 2). In addition, the first two (co-eluting) peaks are aldehydes which are produced by the bacteria in the soil. These aldehydes are

not visible initially due to the large volume of gasoline in the sample, but as the gasoline is degraded, these aldehydes begin to become relatively abundant.

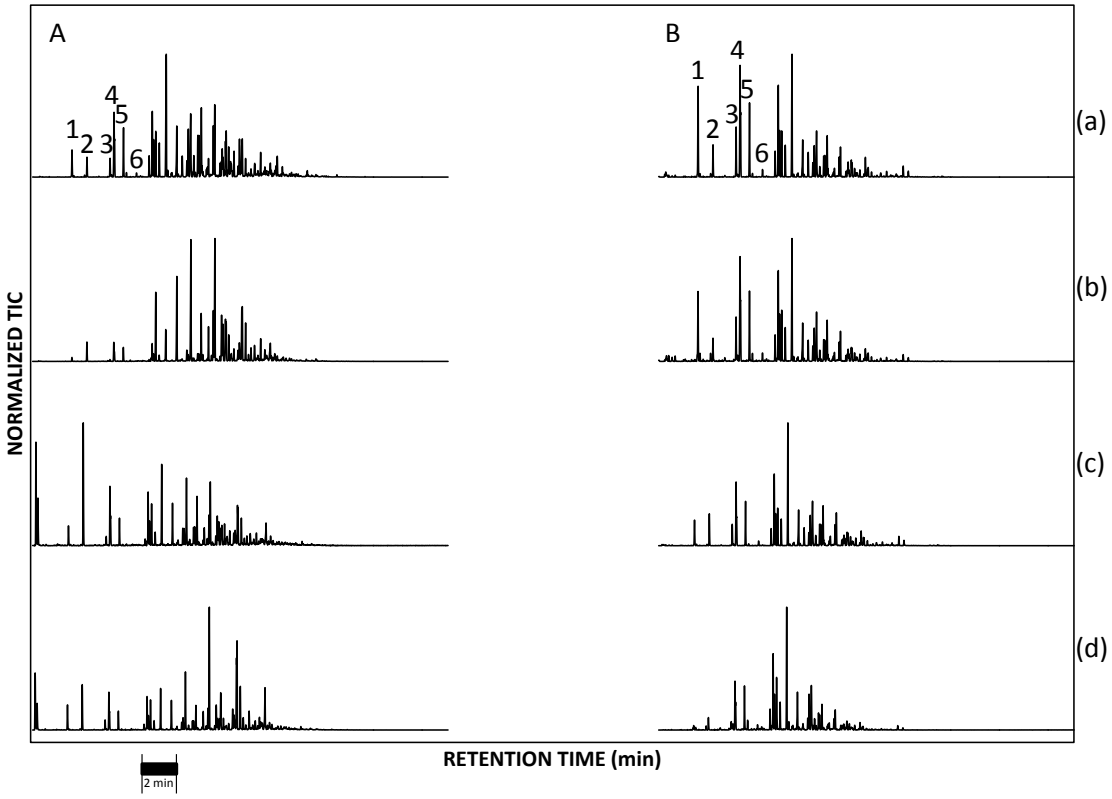


Figure 6-8 TIC of gasoline for site #2 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days. Peaks: (1) toluene, (2) tetrachloroethylene, (3) ethylbenzene, (4) m- & p-xylene, (5) o-xylene, (6) isopropylbenzene.

Figure 6-9 shows the portion of the TIC corresponding to the C_3-alkylbenzenes without treatment (Figure 6-9A) and with treatment (Figure 6-9B) for up to 140 days. Figures 6-6B and 6-9B show that Triclosan can reproducibly preserve gasoline samples. However, microbial degradation can be unpredictable and thus is not always reproducible, particularly in incendiary samples as shown in Figures6- 6A and 6-9A. In Figure 6-9A,

1,2,4-trimethylbenzene as well as the ethyltoluenes and propylbenzene are significantly reduced by 140 days compared to 1,3,5-trimethylbenzene.

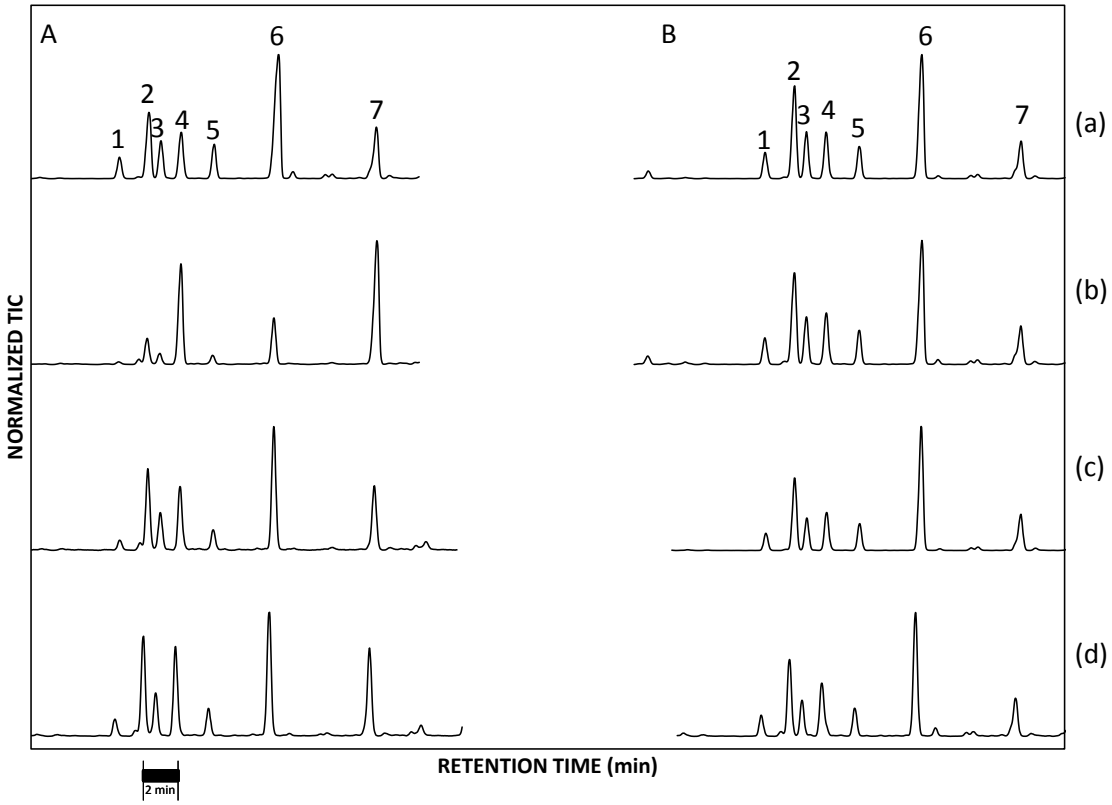


Figure 6-9 C_3-alkylbenzenes in gasoline for site #2 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days. Peaks: (1) propylbenzene, (2) 3-ethyltoluene, (3) 4-ethyltoluene, (4) 1,3,5-trimethylbenzene, (5) 2-ethyltoluene, (6) 1,2,4-trimethylbenzene, and (7) an unknown trimethylbenzene.

Figure 6-10 shows the extracted ion profile corresponding to alkanes (i.e., m/z 57, 71, 85, 99) from gasoline on soil collected from site 2 without treatment (Figure 6-10A) and with treatment (Figure 6-10B). Figure 6-10A shows that untreated samples are subject to significant degradation of the normal alkanes while the n-alkanes are still present even after 140 days in triclosan treated samples (Figure 6-10B).

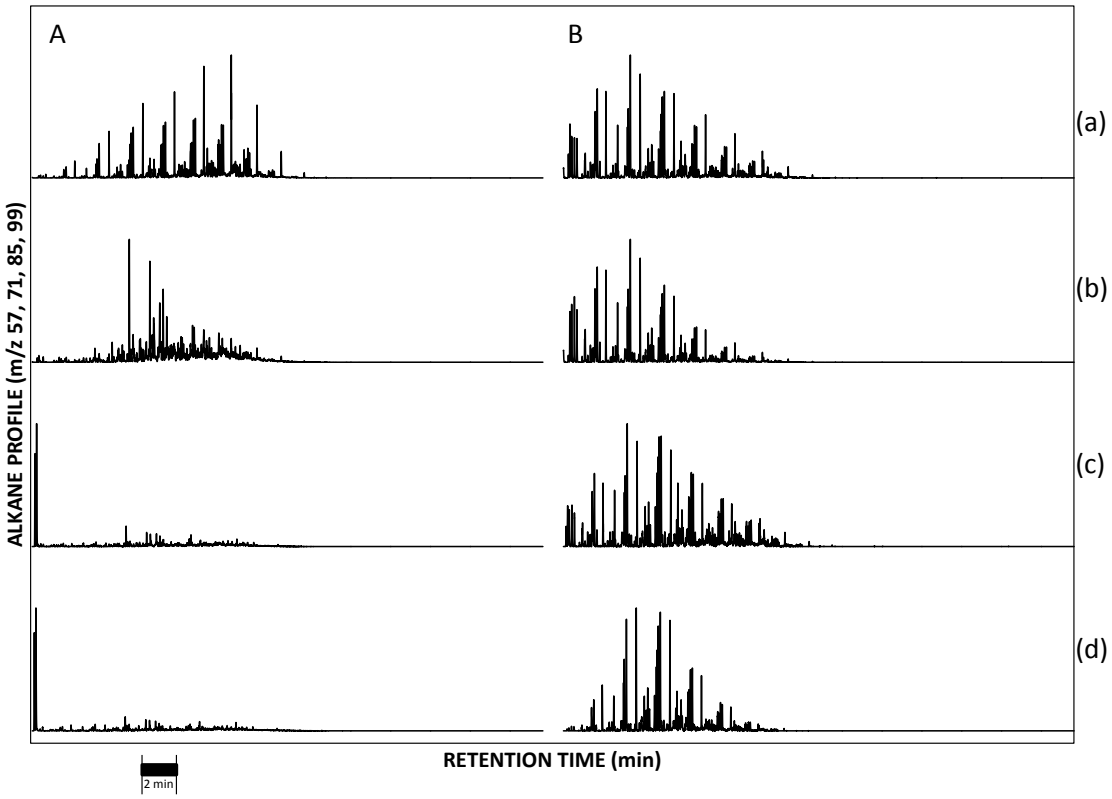


Figure 6-10 Alkane profile of gasoline for site #2 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days.

Figure 6-11 shows the Total Ion Chromatogram (TIC) of gasoline on soil collected from site 3 without treatment (Figure 6-11A) and with treatment using 2% triclosan in 0.2M sodium hydroxide (Figure 6-11B). An overall reduction was observed in the abundance of the lesser substituted alkylbenzenes compared to the C_3-alkylbenzenes, as shown in Figure 6-11A. The severity of degradation in soil collected from site 3 is less than that observed from sites 1 and 2. Variable bacterial populations in soil could explain the differences in degradation of untreated soil samples at the different sites. Figure 6-11B again shows the reproducibility of preservation of gasoline in treated soil samples.

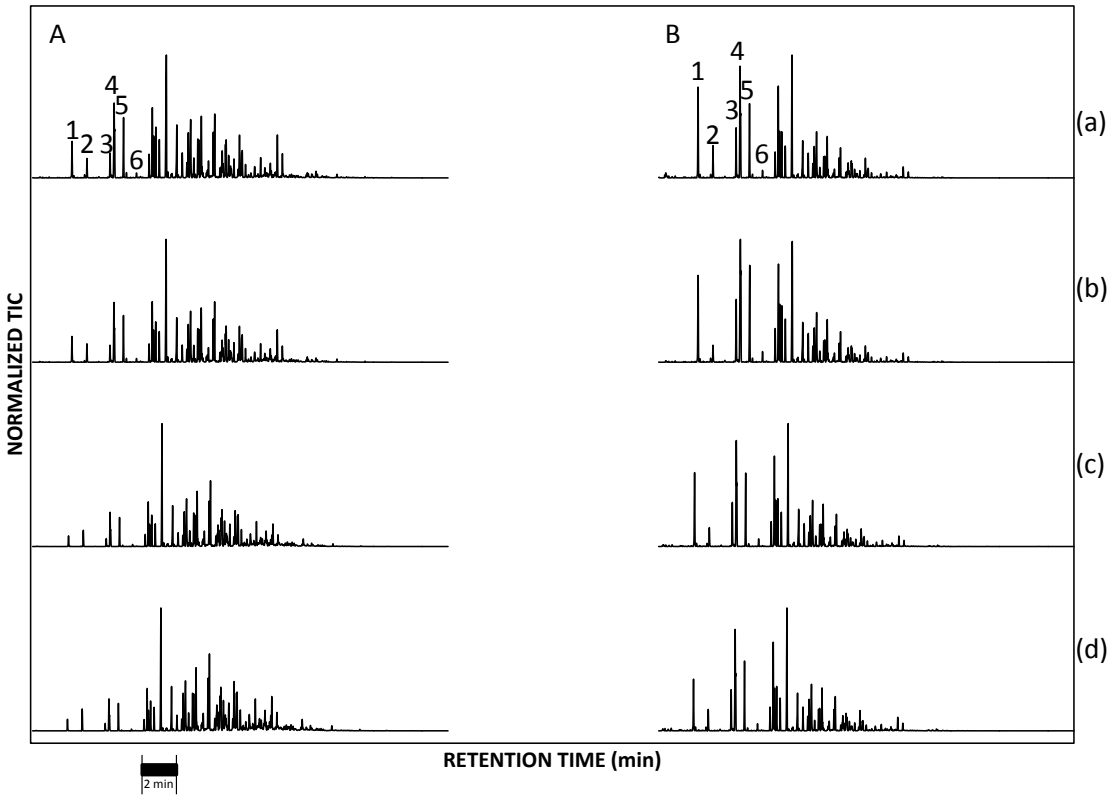


Figure 6-11 TIC of gasoline for site #3 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days. Peaks: (1) toluene, (2) tetrachloroethylene, (3) ethylbenzene, (4) m- & p-xylene, (5) o-xylene, (6) isopropylbenzene.

Figure 6-12 shows the portion of the TIC corresponding to the C_3-alkylbenzenes without treatment (Figure 6-12A) and with treatment (Figure 6-12B) for up to 140 days. It is clear to see that triclosan treated samples are unaffected by bacteria in the soil (Figure 6-12A). However, the untreated samples show little signs of degradation (Figure 6-12B). It is possible that the activity of the bacteria responsible for the degradation of the C_3-alkylbenzenes was reduced compared to those that degrade the normal alkanes.

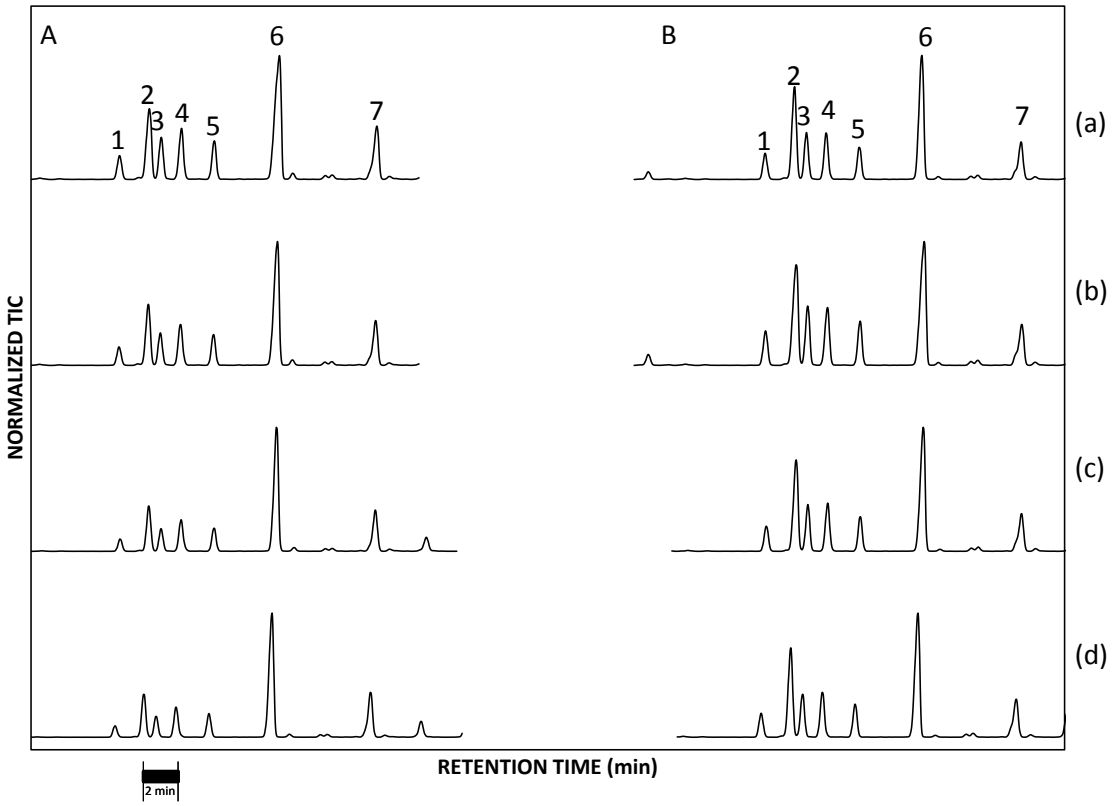


Figure 6-12 C_3-alkylbenzenes in gasoline for site #3 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days. Peaks: (1) propylbenzene, (2) 3-ethyltoluene, (3) 4-ethyltoluene, (4) 1,3,5-trimethylbenzene, (5) 2-ethyltoluene, (6) 1,2,4-trimethylbenzene, and (7) an unknown trimethylbenzene.

Figure 6-13 shows the extracted ion profile corresponding to alkanes (i.e., m/z 57, 71, 85, 99) from gasoline on soil collected from site 3 without treatment (Figure 6-13A) and with treatment (Figure 6-13B). The normal alkanes in the untreated samples are significantly degraded, particularly the earlier eluting normal alkanes (Figure 6-13A). However, the treated samples show no signs of degradation (Figure 6-13B).

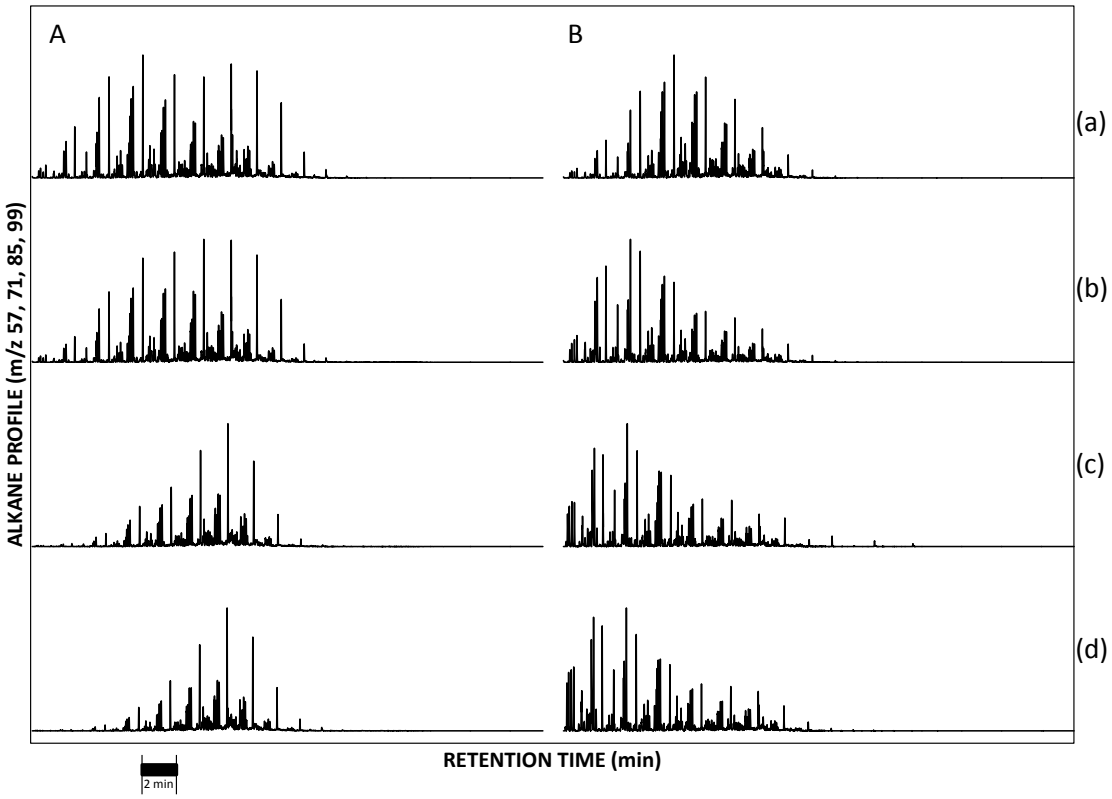


Figure 6-13 Alkane profile of gasoline for site #3 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days.

6.4 Conclusions

Fire debris samples containing soil can exhibit significant microbial activity. In highly degraded samples, the identification and classification of the ignitable liquid residue can be quite difficult. Many household chemicals, while they are fairly inexpensive and easy to use, failed to kill soil bacteria and therefore would not be a good chemical solution for preserving fire debris samples. While cultures showed no bacterial growth during the spectroscopic experiments, passive headspace experiments showed that bacterial degradation begins to occur within 15 days in samples treated with bleach. Additionally, bleach had to be used without dilution and therefore poses a higher risk for

corrosion of the paint can. On the other hand, triclosan dissolved in a sodium hydroxide solution had no greater corrosive effect on the paint can than water. Furthermore, triclosan has been proven to be an effective chemical agent in laboratory and field samples. Triclosan also does not interfere with the analysis of the ignitable liquid residue by a popular ASTM method. Triclosan meets our criterion as an anti-microbial solution and therefore can be applied to the samples upon collection in the field to prevent microbial degradation of the ignitable liquid residues.

CHAPTER 7. FUTURE WORK

Prior to this research, not much was known among fire debris chemists about the effects of microbial degradation of ignitable liquids. Microbial degradation was monitored in many different ignitable liquids from all ASTM classes in potting soil samples. Microbial degradation affects each class in various degrees, however, the most affected classes include gasoline, the petroleum distillates, aromatics, and oxygenated products. Miscellaneous products that contain any of these products are also significantly affected. Microbial degradation of gasoline was also monitored in different types of soil over all seasons. Microbial degradation was greatest in residential soil followed closely by agricultural soil. While the brownfield (industrial) soil samples did show degradation of gasoline, it was significantly less than the other two samples. Seasonal comparison of these soil samples revealed that microbial degradation was most significant in the fall sampling while the summer samples were least affected. Principal Component Analysis was also applied to the peak areas that are most susceptible to microbial degradation. PCA confirmed that the residential and agricultural soils showed the most degradation over 30 days compared to the brownfield soil. PCA showed that microbial degradation was significant in fall, spring, and winter samplings, but not in the summer sampling.

Finally, many chemical solutions were tested for their effectiveness as an antimicrobial solution for preserving fire debris evidence. Triclosan proved to be the most effective in preserving ignitable liquids in soil samples, both in laboratory and field experiments.

Although much has been accomplished, there is more work that can be done to monitor, characterize, and prevent microbial degradation. While microbial degradation has been shown to occur in soil due to a high bacterial load, microbial degradation of ignitable liquids has not been studied in wood samples, particularly rotting wood which may also contain a high bacterial load. Biologists and environmental scientists are quite familiar with bacterial and fungal species that co-exist with or decompose dead wood [94-100]. While degradation of ignitable liquids on rotting wood has not been studied, degradation studies of polycyclic aromatic hydrocarbons by bacteria that also degrade wood has been studied [94]. If bacteria and fungi are capable of degrading PAHs, it is plausible that other hydrocarbons such as those found in ignitable liquids would also be degraded by bacteria found on decomposing wood. Microbial degradation should be studied in a variety of wood samples including clean wood chips, wood chips collected from outdoor flower beds, and rotting wood chopped up and collected from dead trees. Wood, as other fire debris substrates often do, may give off volatile compounds that could interfere with the analysis of fire debris samples so it is important to analyze non-spiked wood samples. It would also be interesting to add to this study a comparison of microbial degradation in soil samples to determine which substrate poses the highest threat of degradation to the ignitable liquid residue.

An ignitable liquid that contains oxygenated compounds is somewhat problematic for analysis by passive headspace analysis, particularly in porous substrates such as soil.

The adsorbent material typically used in passive headspace analysis of ignitable liquids is activated charcoal. Volatile molecules adsorb to the charcoal strip through London dispersion forces [43]. Since the interaction between the molecule and the strip are merely physical, other molecules that have a stronger affinity for the activated charcoal can displace molecules with a weaker affinity [42, 43]. Oxygenated compounds have a higher affinity for a more polar substrate like polyethylene glycol (PEG). Supelco offers PEG fibers for solid phase microextraction (SPME) of the analysis of oxygenated compounds, but PEG strips for passive headspace could also be fashioned. By fashioning PEG strips to the same size as carbon strips, a comparison could be made between the recoveries of oxygenates via PEG and the activated charcoal strips. The method should also be optimized prior to degradation studies since PEG has not been utilized in passive headspace analysis.

In our degradation studies in collaboration with Ball State University we determined that microbial degradation was significantly less in the summer soil samples. It is possible that the summer soil was fertilized by the land owners prior to soil collection. Fertilized soil likely contains significantly more TOC than non-fertilized soil, giving the bacteria an alternate energy source. Furthermore, fertilized soil may contain other micronutrients that would support other physiological functions, such as a nitrogen source. A study should be conducted to test this theory wherein fertilized and non-fertilized soil is spiked with gasoline and monitored for microbial degradation. If our hypothesis is true, the fertilized soil will show little to no degradation while the non-fertilized soil will show significant degradation.

While triclosan has proved to be an effective anti-microbial solution for the preservation of ignitable liquid residues, it remains controversial as an antibacterial in hand soaps, lotions, and the like. According to the FDA, triclosan does not possess any hazards to human health [101]. However, scientific studies in animals have shown that hormone regulation is altered by exposure to triclosan. The FDA continues to participate in scientific and regulatory reviews of the effects of triclosan but does not necessarily recommend consumers to change to products that do not contain triclosan [101]. While this controversy alone is not enough to avoid using triclosan as a preservative of ignitable liquid residues in fire debris, having an alternative that is just as effective as triclosan would be beneficial. We believe based on initial microbiological studies for the effectiveness of various chemicals that the active ingredients in Lysol products could provide that alternative. Lysol 4-in-1 bathroom cleaner contains various alkyl dimethyl benzyl ammonium chlorides (e.g. benzalkonium chloride) and Lysol All Purpose cleaner contains citric acid as the active ingredient. Microbiological studies should be conducted in soil samples to determine at what concentration these active ingredients are effective in killing all bacteria. Once a concentration has been determined for these compounds, passive headspace studies should be conducted to show the effectiveness of these compounds in an aqueous solution over time at preserving gasoline and other ignitable liquids.

Previous research has shown that the pathway that is responsible for alkane degradation in bacterial strains such as Pseudomonas is turned off when the bacteria have sufficient nutrients that are preferred over alkanes [102]. Exposure to growth media containing various carbon sources were studied for the degradation of alkanes by

different strains of alkane degrading bacteria. These studies could be applied in studies for the prevention of microbial degradation of ignitable liquids in fire debris samples containing soil as the matrix. Soil samples spiked with a gasoline, n-alkane product, and an aromatic product should be studied with and without treatment with various growth media. Growth media to be studied should include minimal media containing different carbon sources such as citric acid and lactic acid, as well as a more nutrient dense media such as Tryptic Soy broth. The n-alkane product and the alkanes in gasoline should be preserved in nutrient rich samples. The pathway responsible for the degradation of aromatics may differ from that of the alkanes. However, our hypothesis in previous degradation studies indicates that a nutrient rich environment would prevent microbial degradation of alkanes as well as aromatics.

Our previous studies to compare degradation over different soils and seasons were limited to soil and climate in northern Indiana. However, microbial degradation could vary among other soils in areas of the country and the world that experience different climates. As we saw in our field experiment where degradation was not apparent in soil samples collected during an unseasonably hot and dry summer. The soil most likely experienced a reduction in bacterial activity due to these unseasonable conditions. While these types of conditions may be typical in some states or countries, bacterial populations could vary which could be used to compare microbial degradation or lack thereof in a variety of soils. This information could be used to create a database of degraded ignitable liquids in various seasonal conditions and soil types. These studies should also include water content measurements, as this was not done in our studies. Water content is important for bacterial growth in aerobic soil bacteria. So experiments that look at the

moisture level of the soil may shed some light on the bacterial activity in the soil. Additionally, more work should be conducted to look at trends in soil chemistry that affects bacterial growth. Specifically, PCA could be used to determine what nutrients dominate bacterial growth and what other compounds found in soil, such as heavy metals, impede bacterial growth.

Another interesting study is to determine if the bacteria do have a preference to even n-alkanes over odd n-alkanes. Initial studies conducted in our laboratory suggested a preference for the n-alkanes in heavy petroleum distillates. However, this hypothesis was not proven. This is because all of the liquids in our studies containing normal alkanes were not of equal concentration and no internal standard was used. A study should be conducted where a standard solution containing equal molar concentrations of n-alkanes from C_7 to C_{20} is spiked onto soil. An internal standard such as hexamethylbenzene should also be spiked onto the soil. Degradation monitored over time may reveal that even n-alkanes are significantly more degraded than odd n-alkanes.

Quantitative analysis is also something that should be pursued. Quantitative analysis would be beneficial in many of the previously mentioned studies. While quantitative analysis is not necessary in current fire debris analyses, from a research perspective it would be useful in proving hypotheses discussed in this work. However, quantitation in fire debris samples is far from simple. Generally, fire debris samples contain significantly more ignitable liquid residues than can adsorb onto the activated charcoal strip that is currently used. Due to cost, increasing the size of the strip to accommodate the concentrated samples is not practical. Sigman and co-workers suggested that one way to overcome this challenge is to heat the sample prior to sampling

to evenly distribute the ignitable liquid residue throughout the sampling container [43]. Once the sample has been cooled back to room temperature, a subsample could then be taken for analysis. Quantitation in microbe-rich substrates such as soil is also difficult due to the need of a surrogate internal standard that will not be subject to microbial degradation. Our lab determined that hexamethylbenzene is a suitable surrogate internal standard for use in fire debris samples containing a soil matrix. A study should be conducted where in soil samples are spiked with various ignitable liquids and hexamethylbenzene as an internal standard. The samples should then be heated to ensure the ignitable liquid is evenly distributed. Then a small subsample can then be analyzed by passive headspace and quantitated. The determined concentration can then be compared back to the original concentration to determine the accuracy of this method.

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APPENDICES

Appendix A Chromatograms of Degraded Ignitable Liquids from UCF

Approximately 100g of potting soil was spiked with 20µL of the ignitable liquid. The samples were allowed to age up to 21 days. On the day of analysis a whole carbon strip was suspended into the headspace of the can on a prebaked paper clip using a nylon string. The cans were baked at 65°C for 16h. Upon cooling, the strips were cut in half. One half of the strip was stored and the other half was extracted with 600µL of pentane. All data was acquired using an Agilent 6890 Gas Chromatograph with an Agilent 5975 Mass Spectrometer. The GC was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 microns). The carrier gas was helium with a flow rate of 1mL/min. The method utilized an inlet temperature of 250°C, 1 µL injection volume, and a 20:1 split ratio. The default oven temperature program started at 40°C for 2 minutes, ramped to 280°C at 10°C/min. and held for 3 minutes. The MS parameters included a solvent delay of 2 minutes. Additionally, the scan range was 40-300m/z.

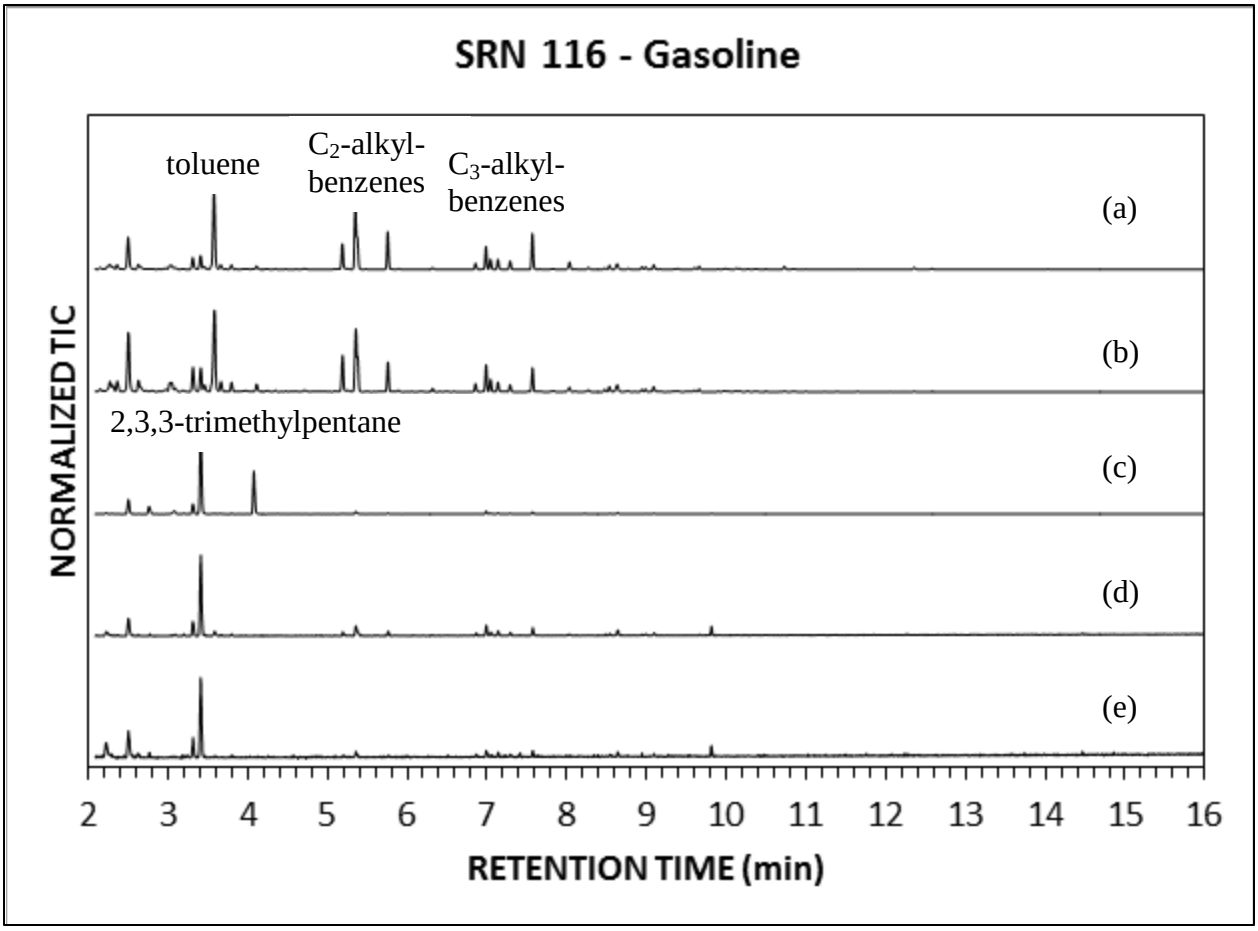

Figure A-1 Microbial degradation of gasoline, SRN116: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21.

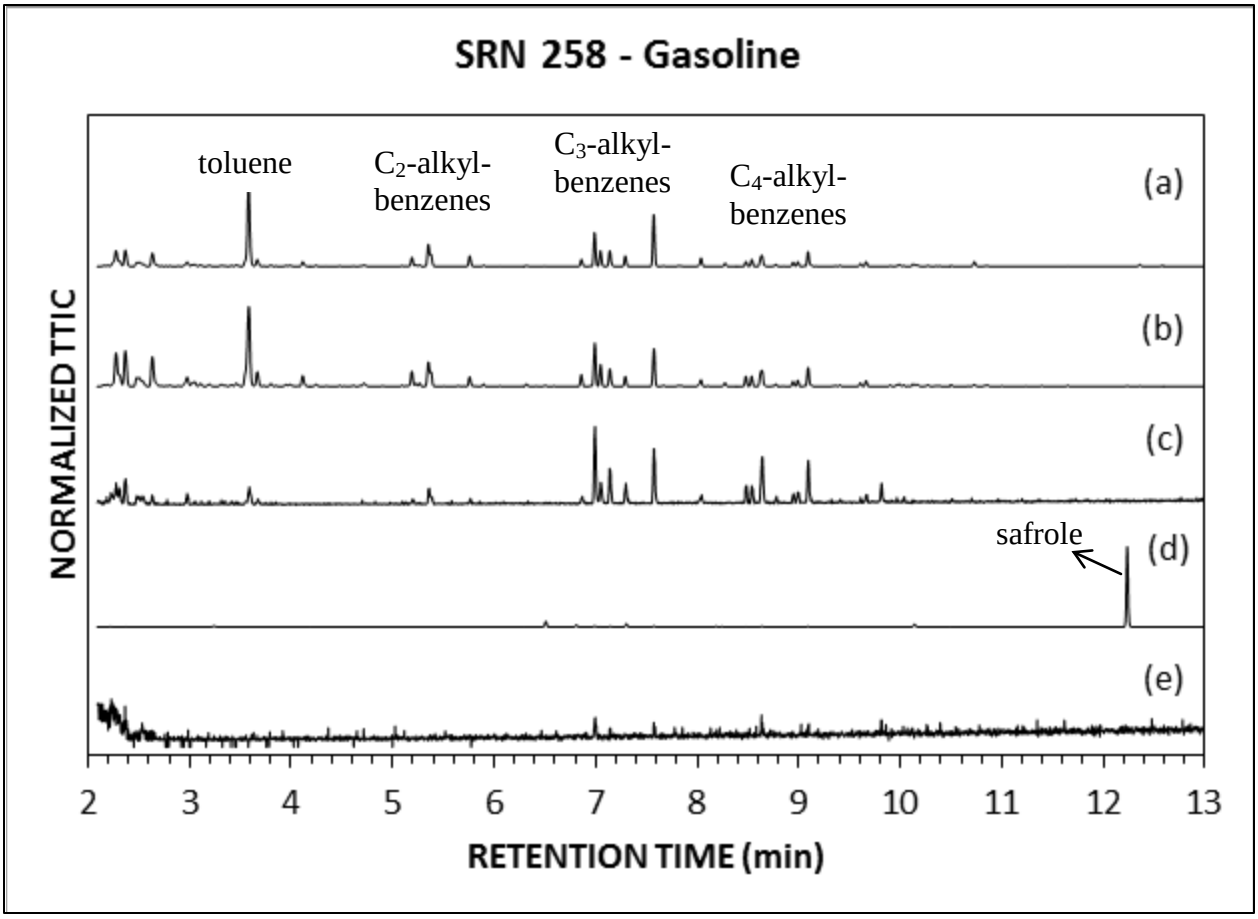

Figure A-2 Microbial degradation of gasoline, SRN258: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

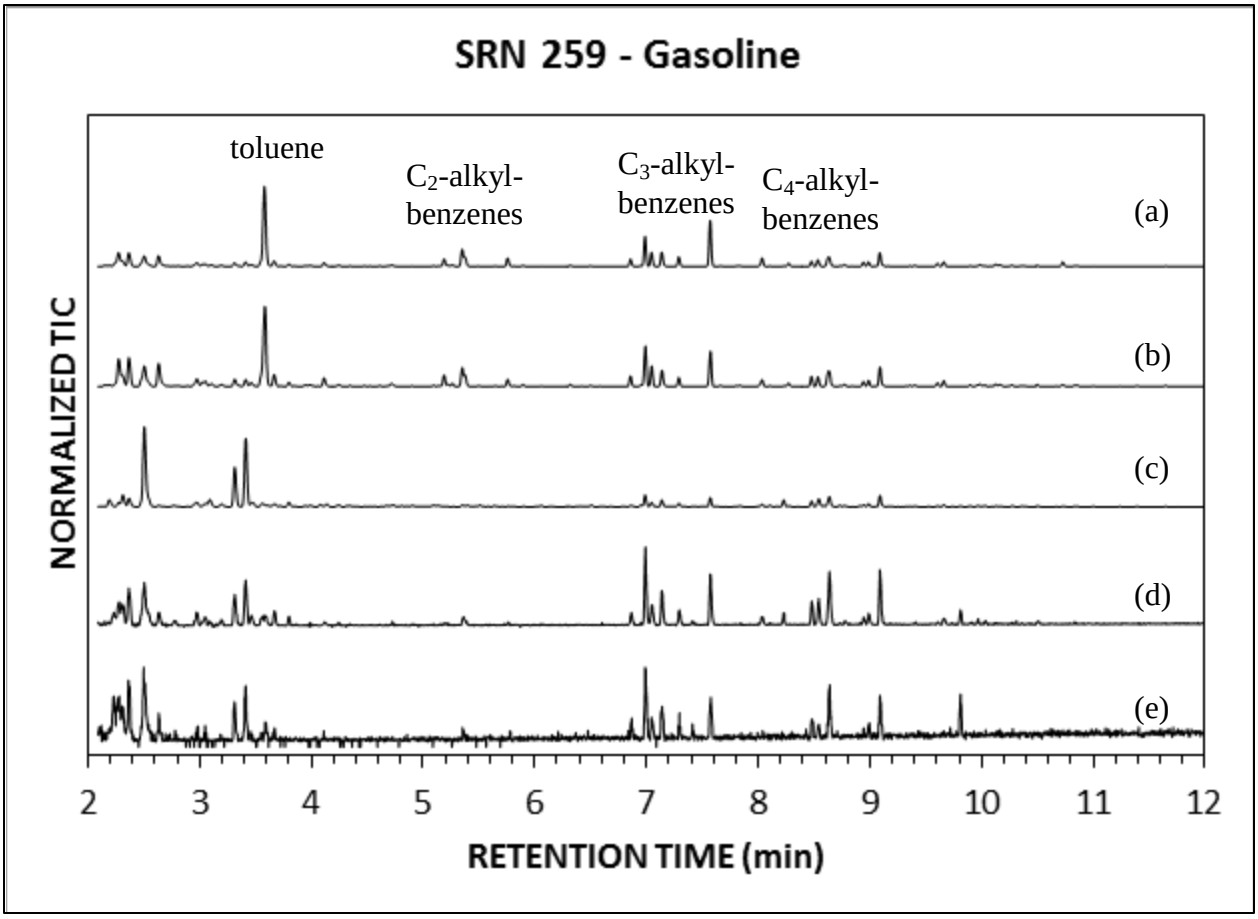

Figure A-3 Microbial degradation of gasoline, SRN259: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

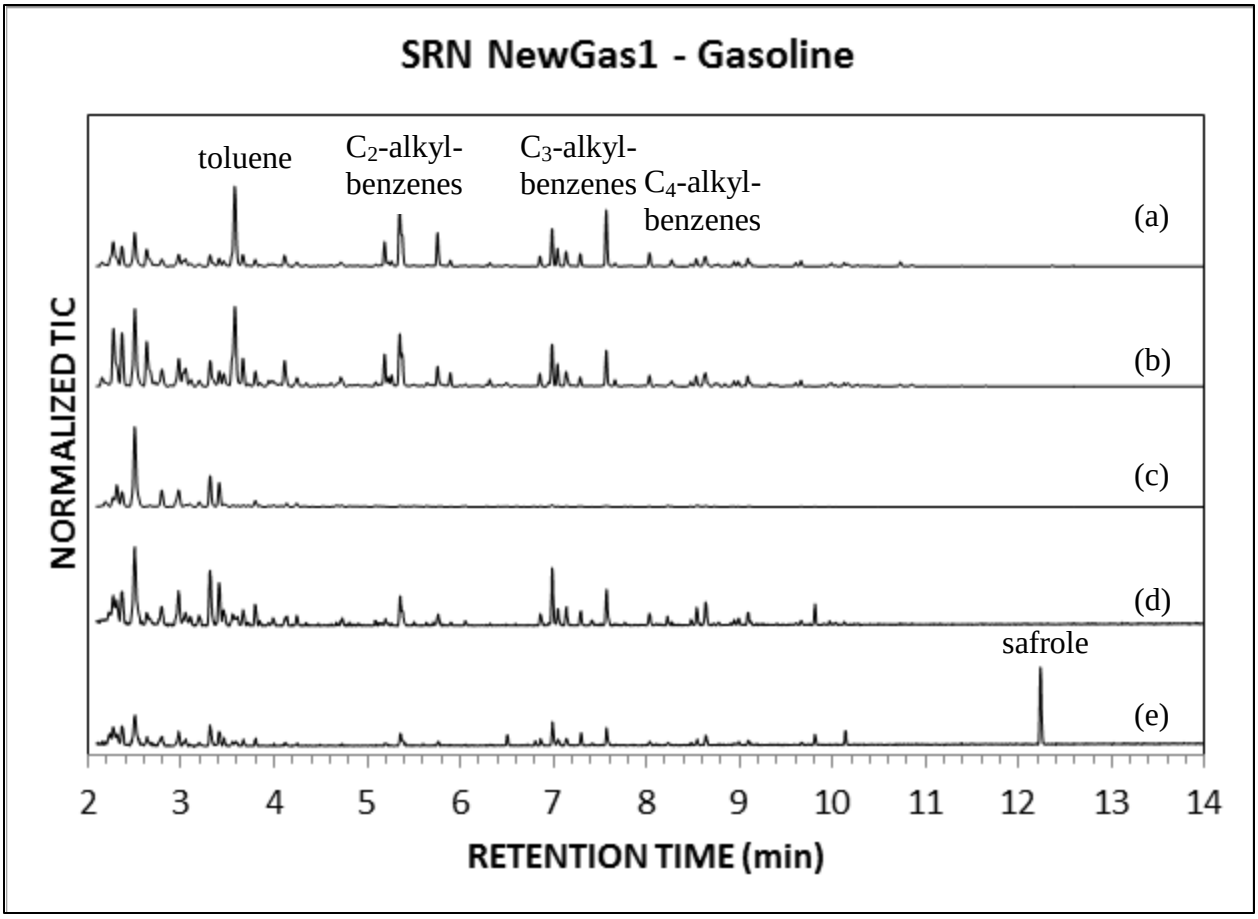

Figure A-4 Microbial degradation of gasoline, SRN NewGas1: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

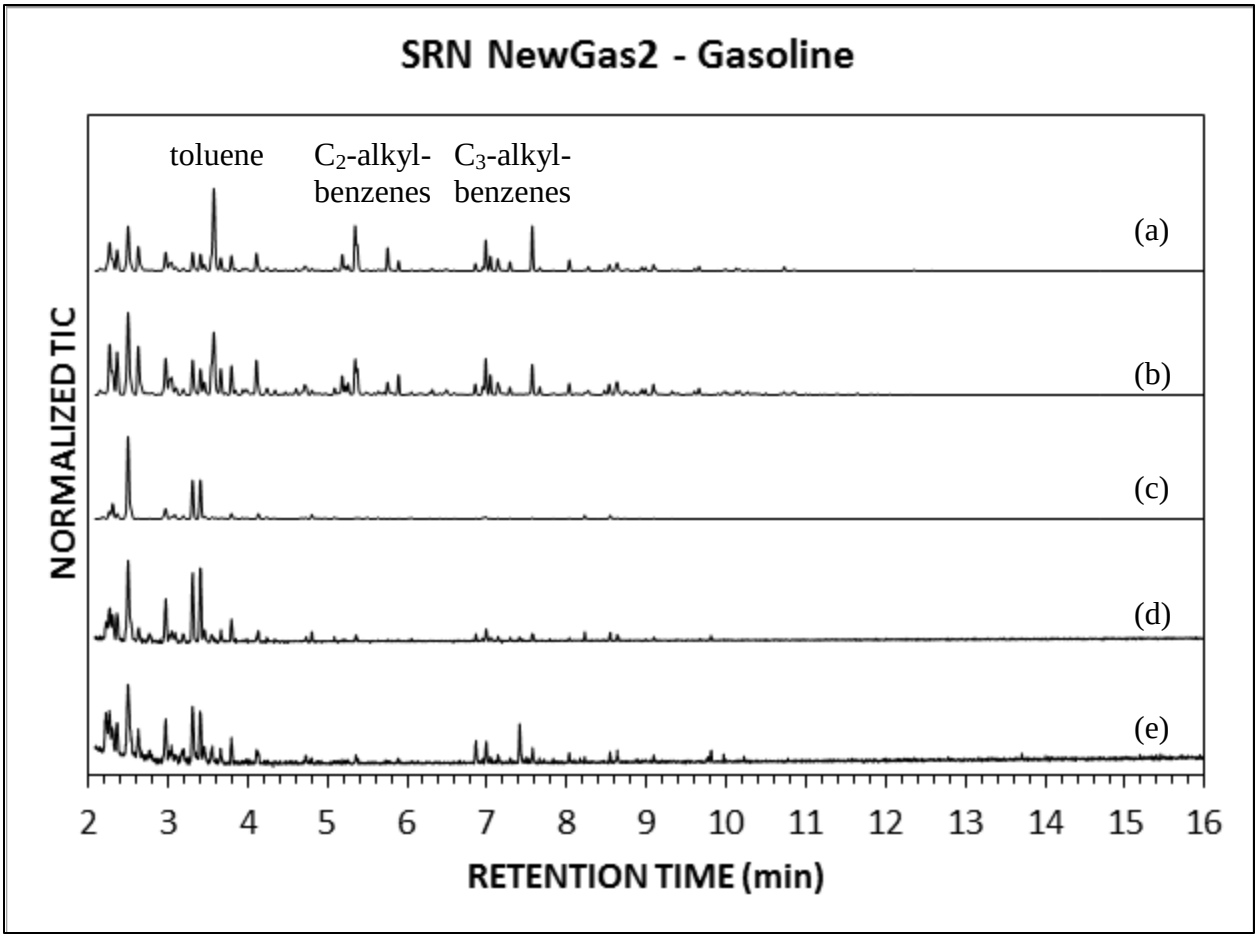

Figure A-5 Microbial degradation of gasoline, SRN NewGas2: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

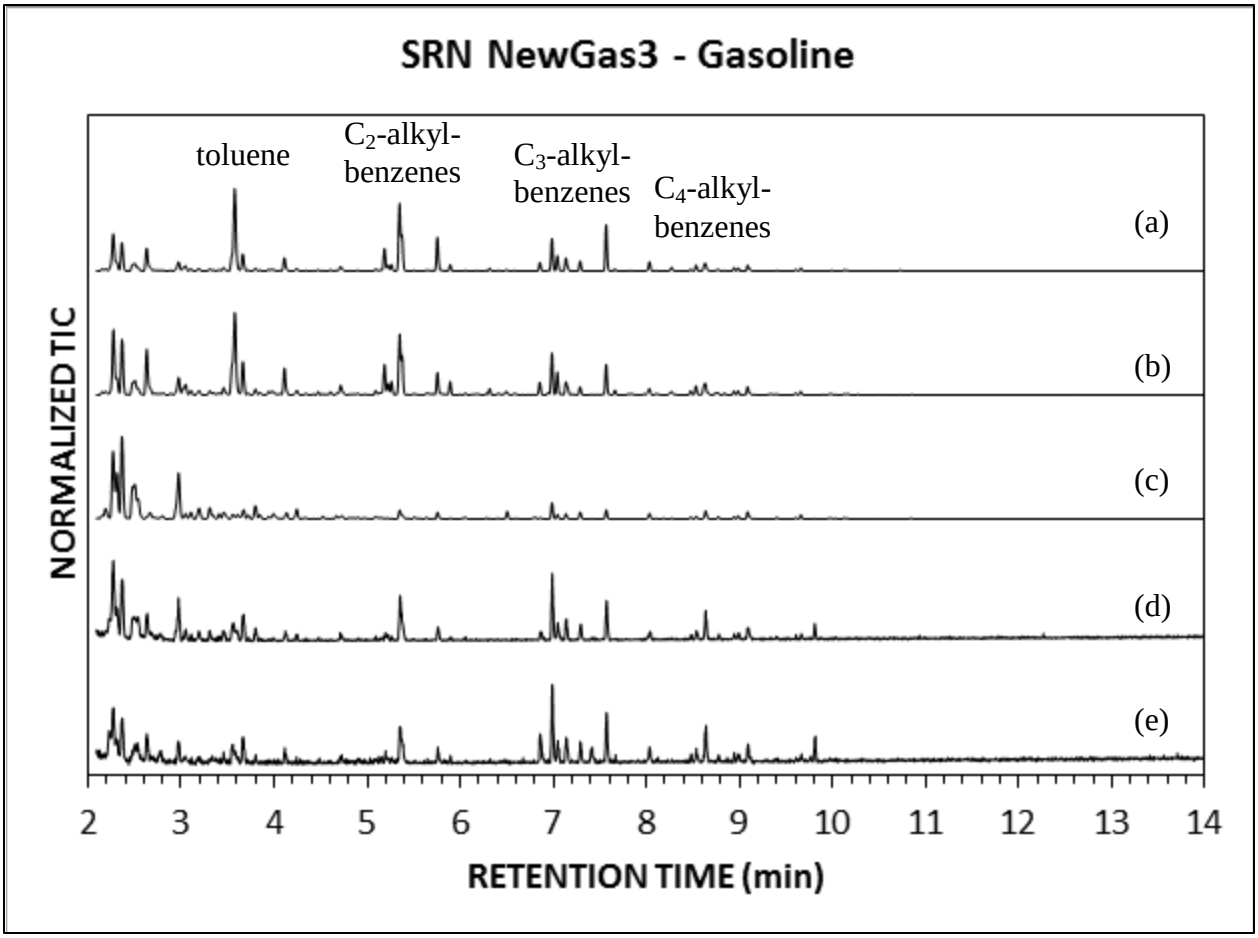

Figure A-6 Microbial degradation of gasoline, SRN NewGas3: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

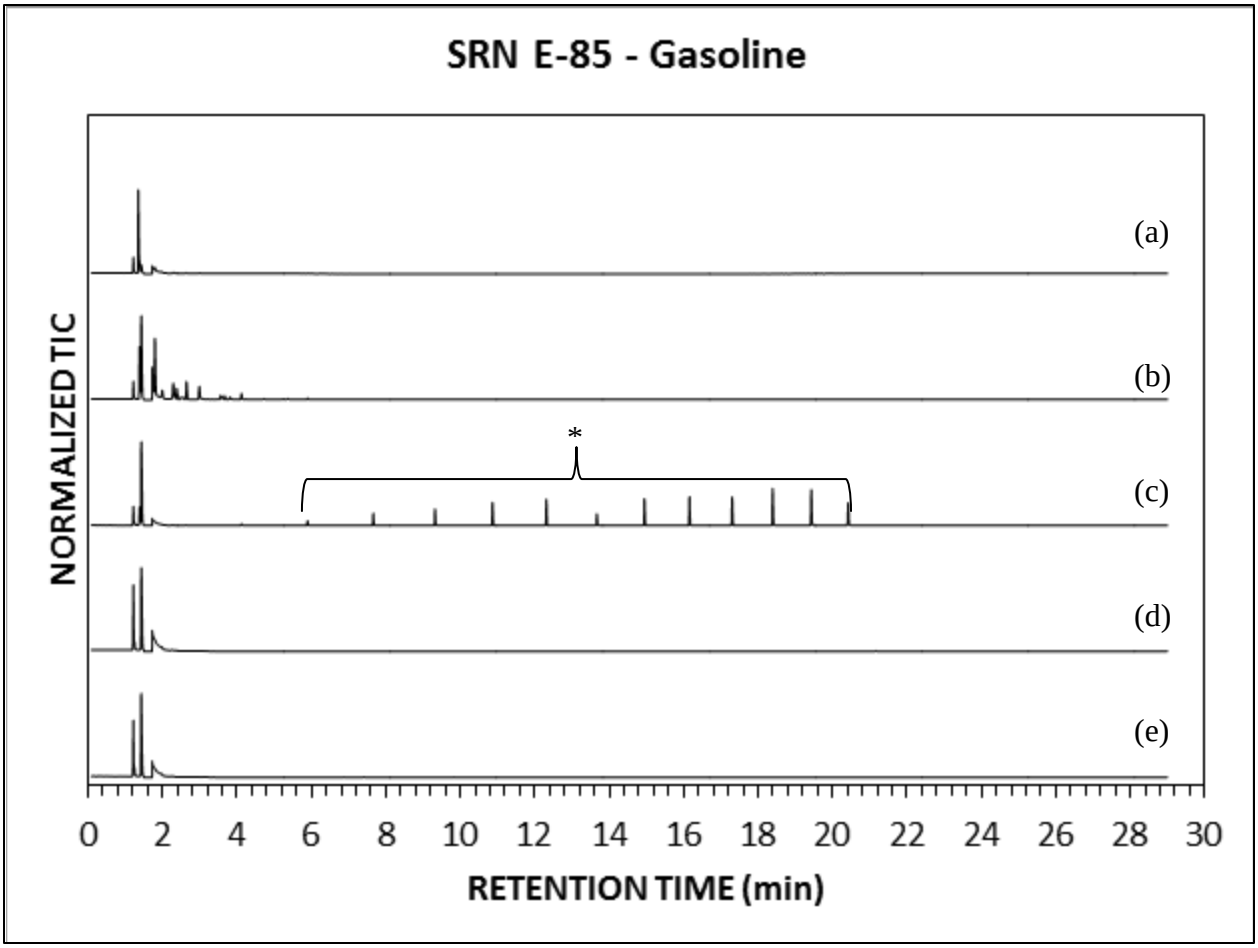

Figure A-7 Microbial degradation of gasoline, SRN E-85: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21. * These peaks are n-C_9 to n-C_{20} and are likely due to contamination or carry-over.

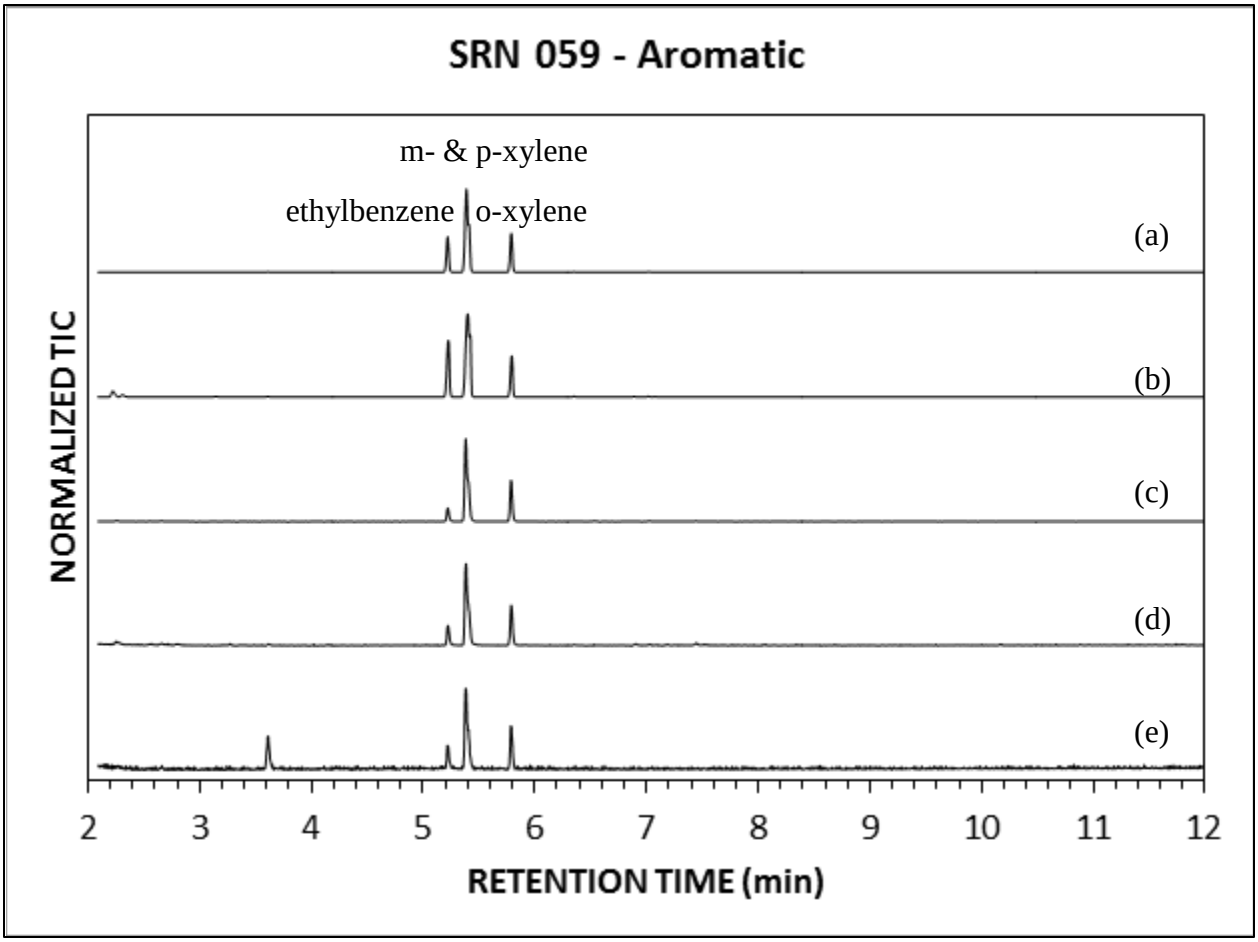

Figure A-8 Microbial degradation of an aromatic product, SRN059: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21.

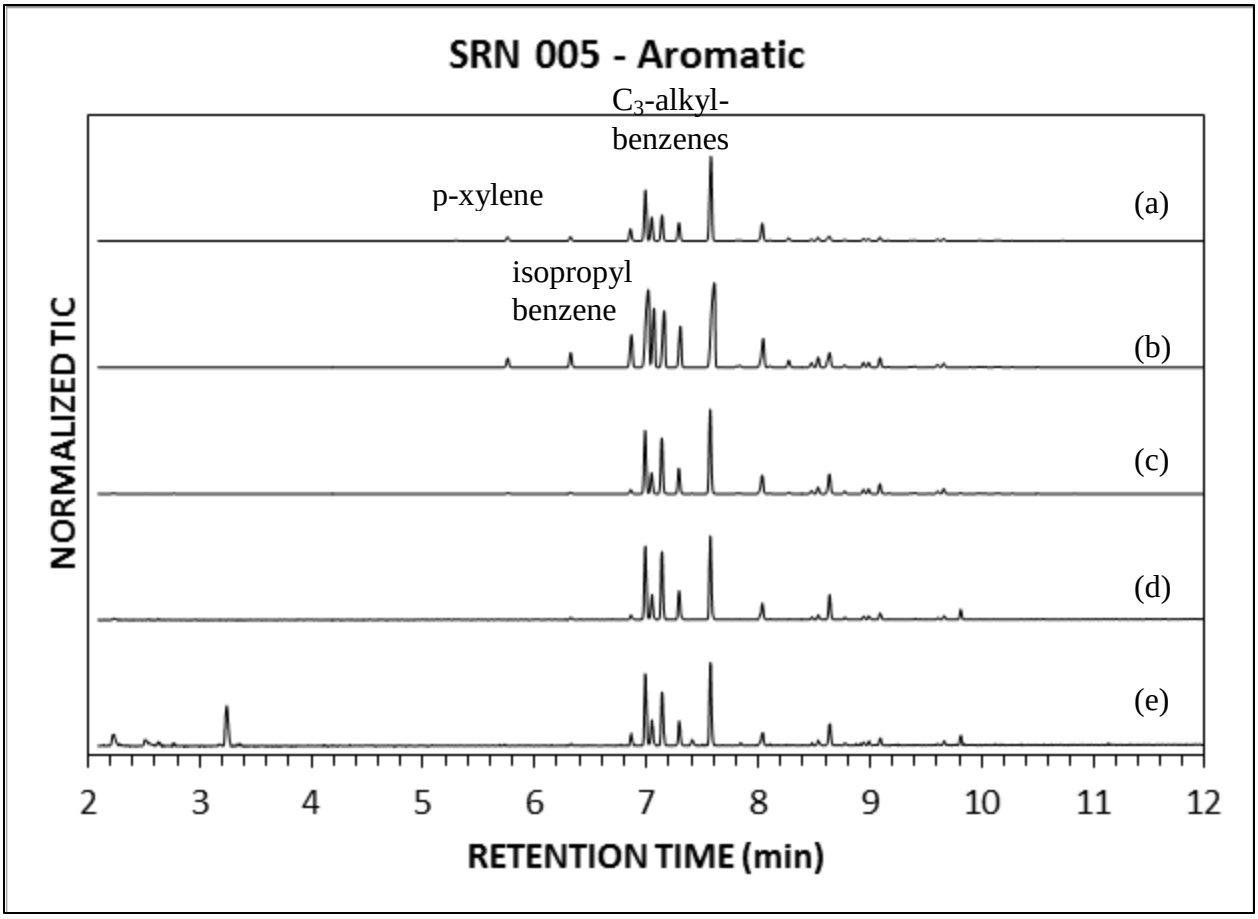

Figure A-9 Microbial degradation of an aromatic product, SRN005: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21. The C_3-alkylbenzenes include: propylbenzene, 3-ethyltoluene, 4-ethyltoluene, 1,3,5-trimethylbenzene, 2-ethyltoluene, and 1,2,4-trimethylbenzene consecutively.

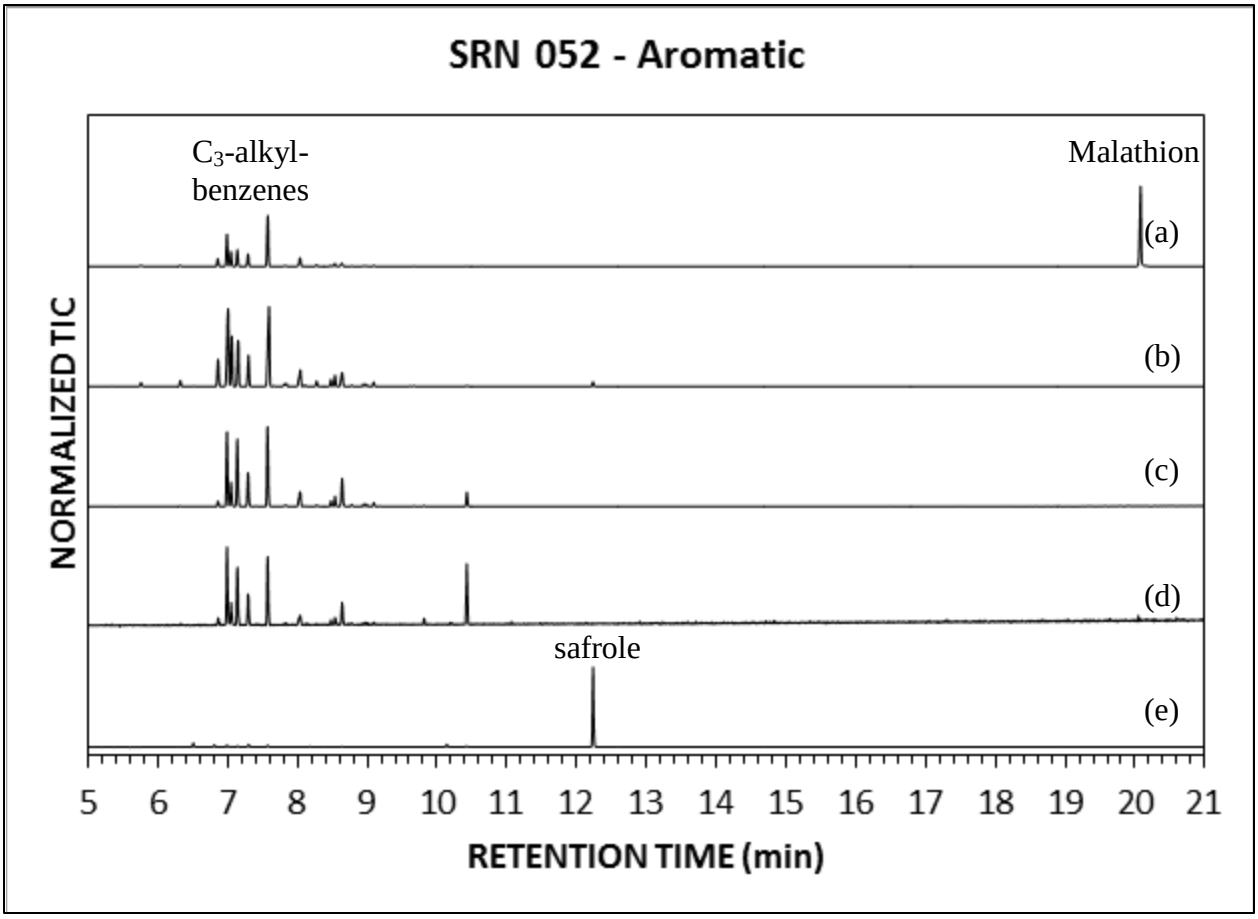

Figure A-10 Microbial degradation of an aromatic product, SRN052: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

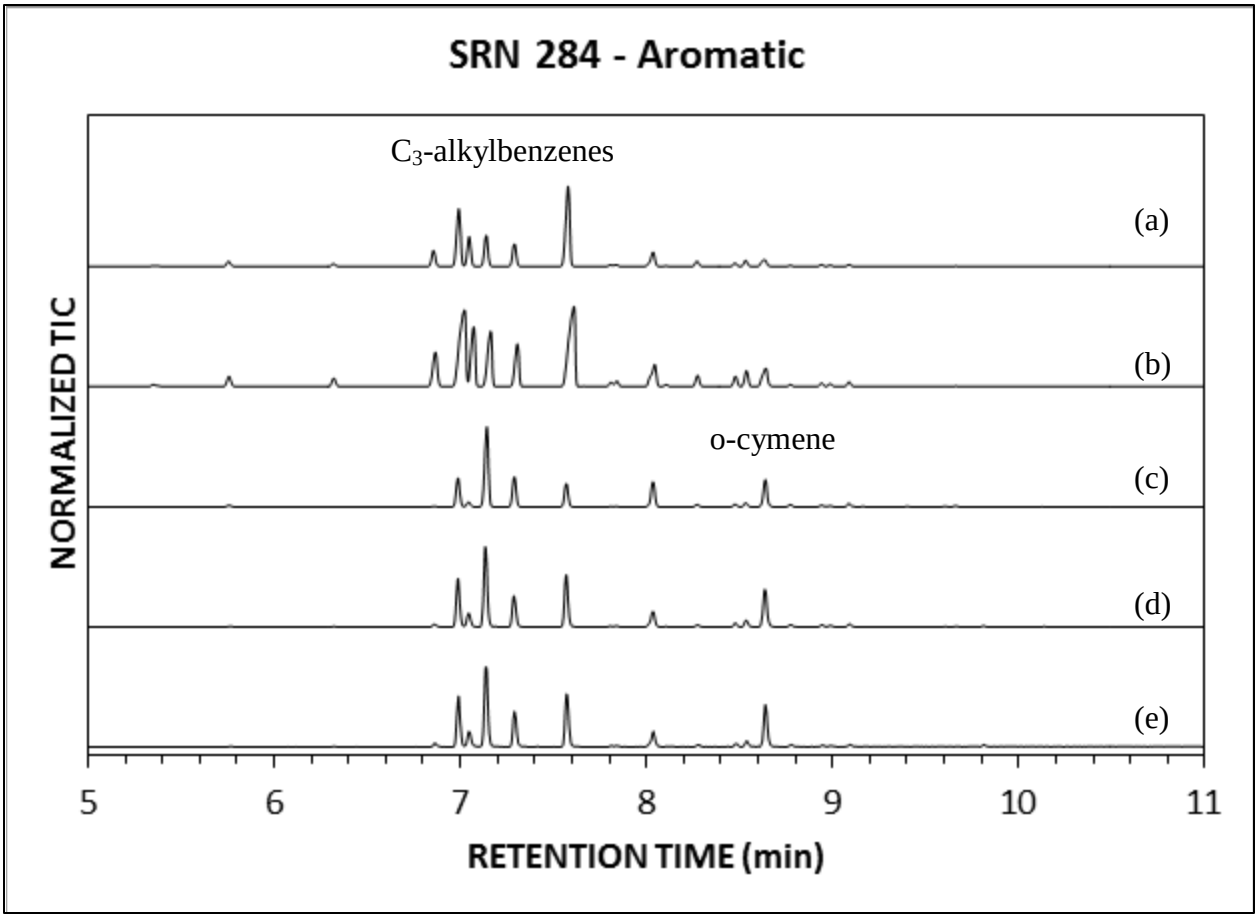

Figure A-11 Microbial degradation of an aromatic product, SRN284: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

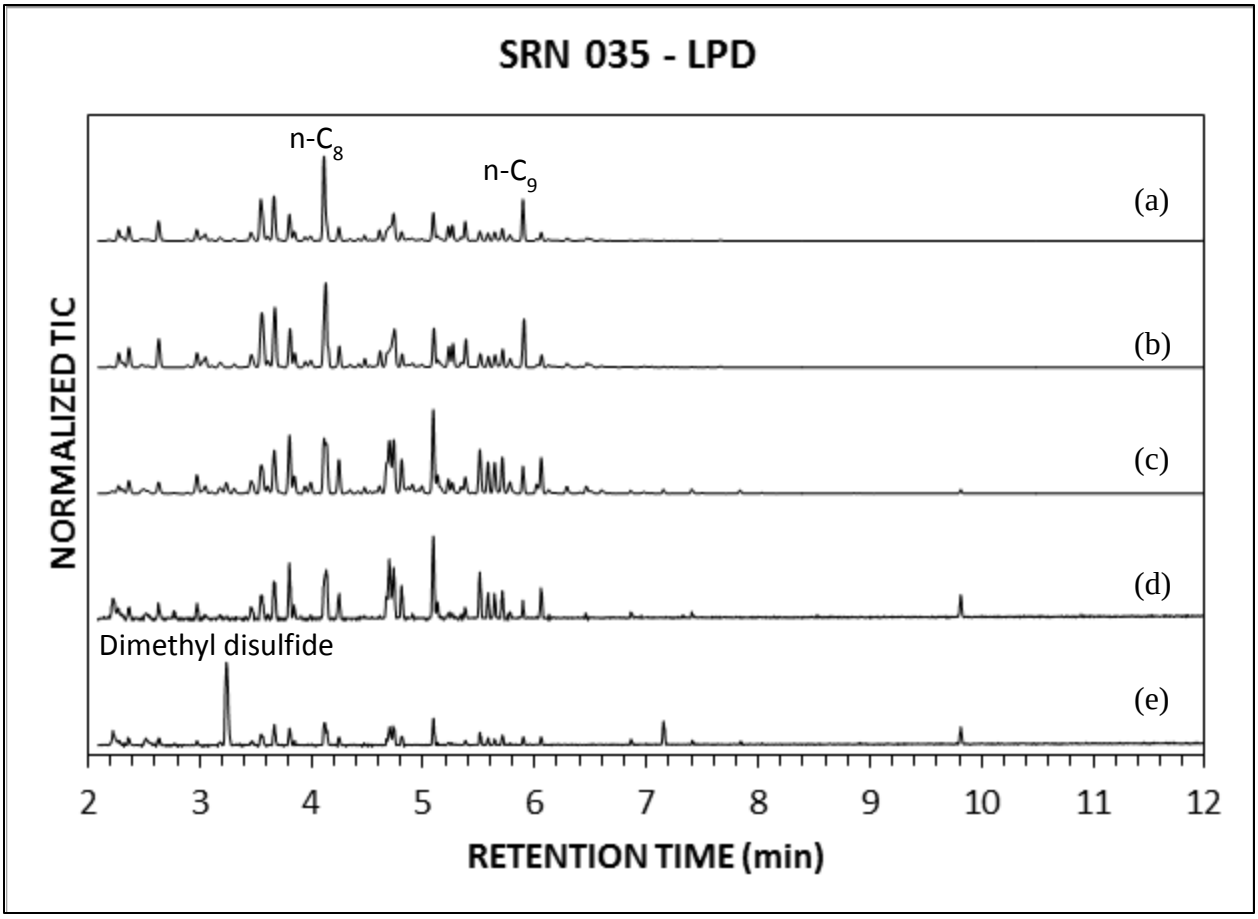

Figure A-12 Microbial degradation of a light petroleum distillate, SRN035: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

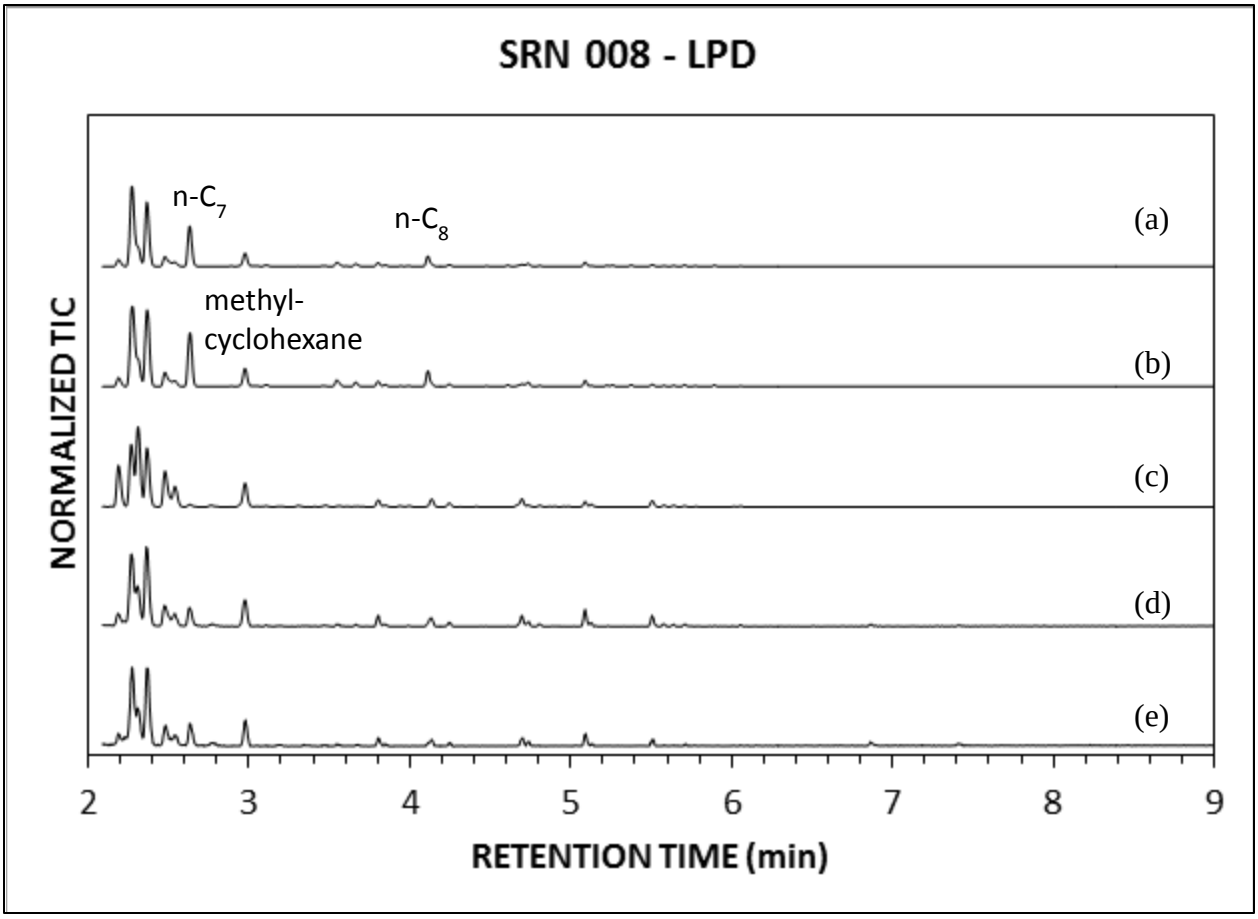

Figure A-13 Microbial degradation of a light petroleum distillate, SRN008: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

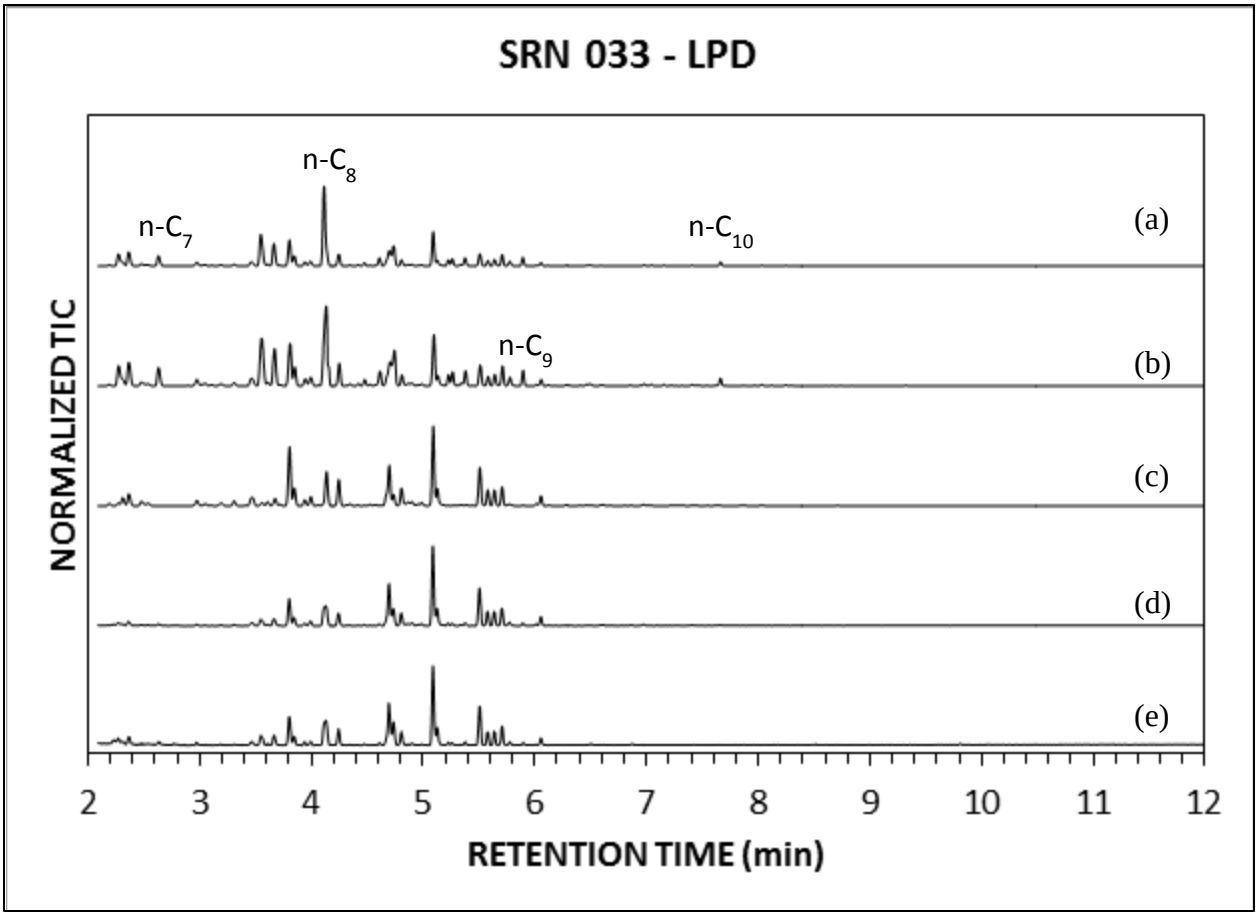

Figure A-14 Microbial degradation of a light petroleum distillate, SRN033: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

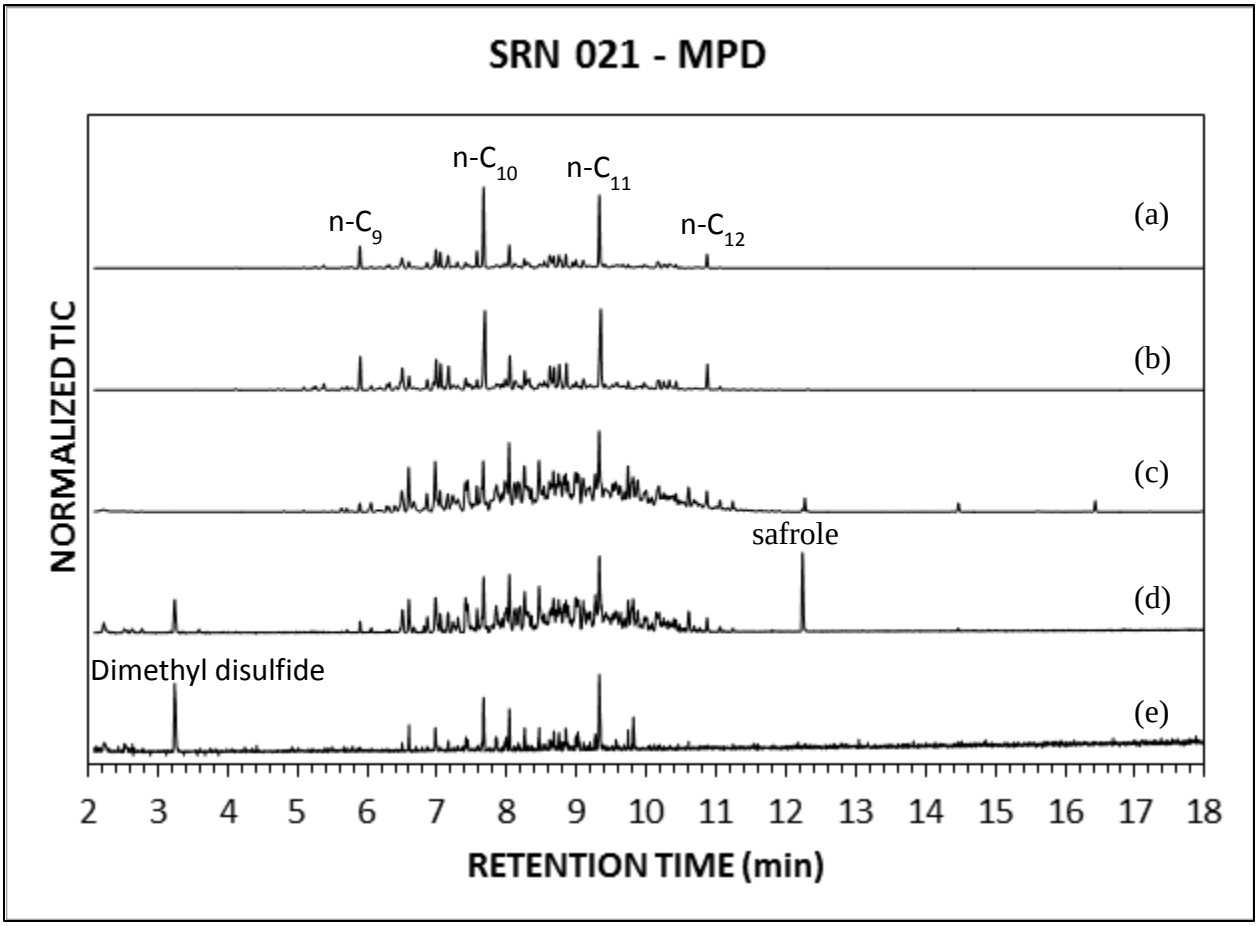

Figure A-15 Microbial degradation of a medium petroleum distillate, SRN021: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21.

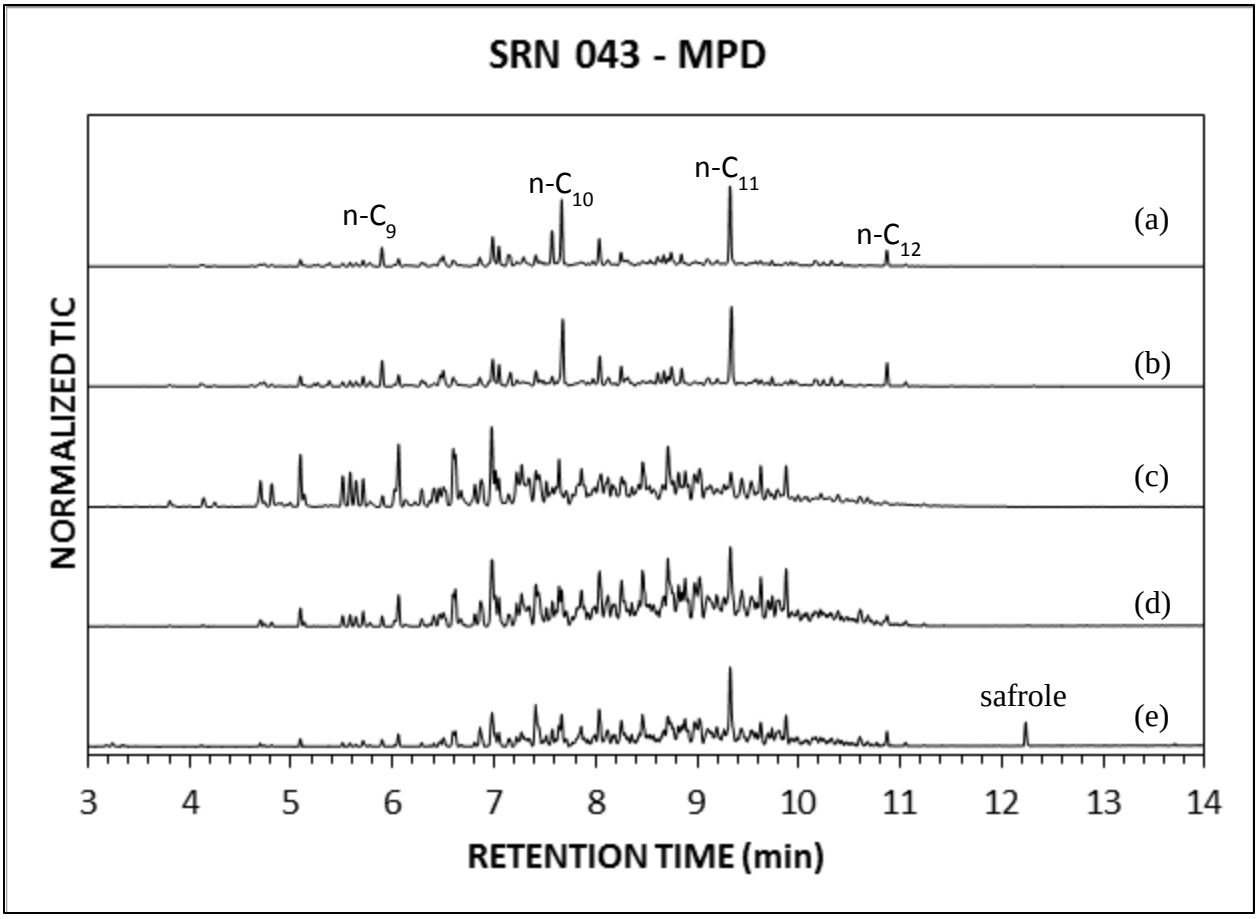

Figure A-16 Microbial degradation of a medium petroleum distillate, SRN043: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

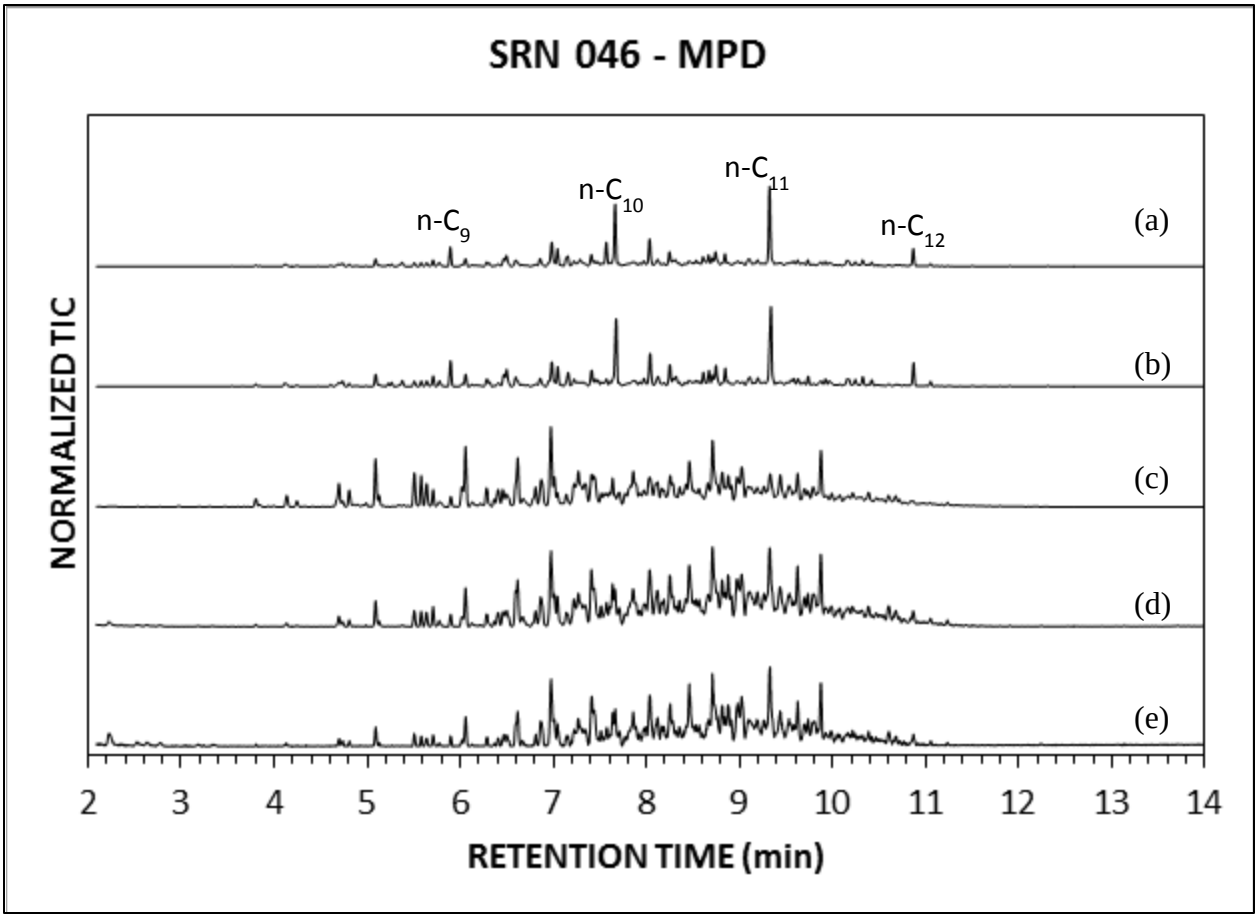

Figure A-17 Microbial degradation of a medium petroleum distillate, SRN046: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

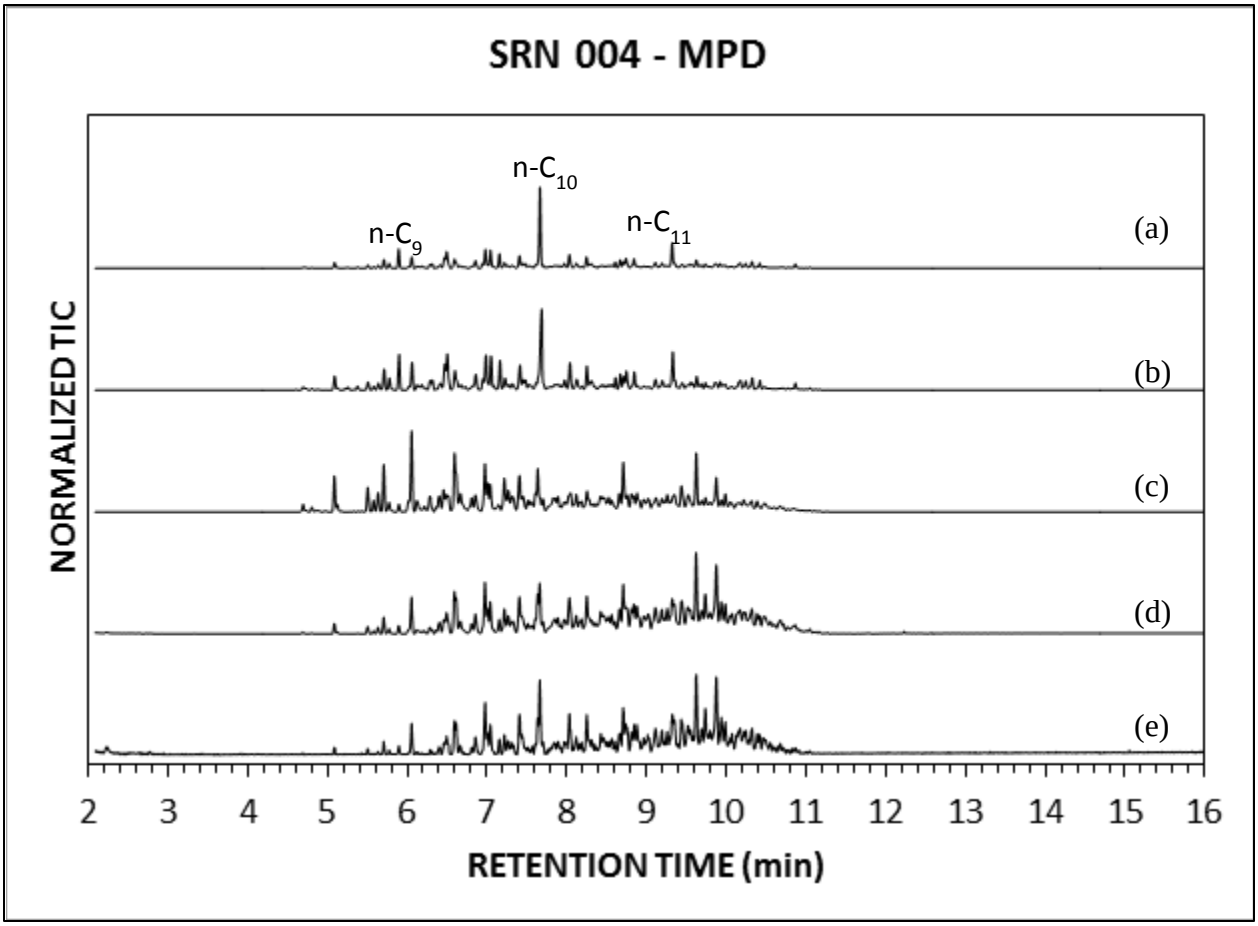

Figure A-18 Microbial degradation of a medium petroleum distillate, SRN004: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

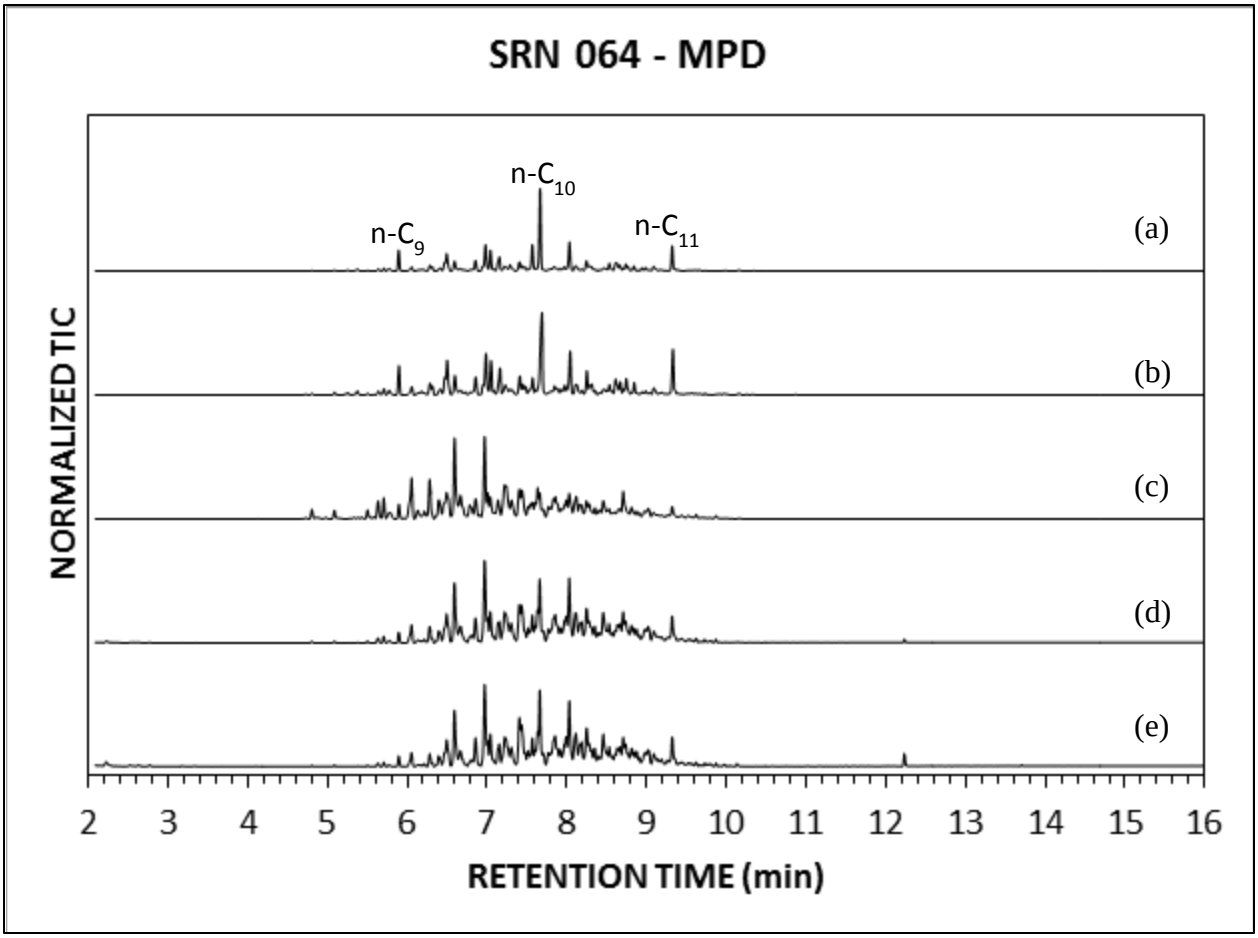

Figure A -19 Microbial degradation of a medium petroleum distillate, SRN064: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

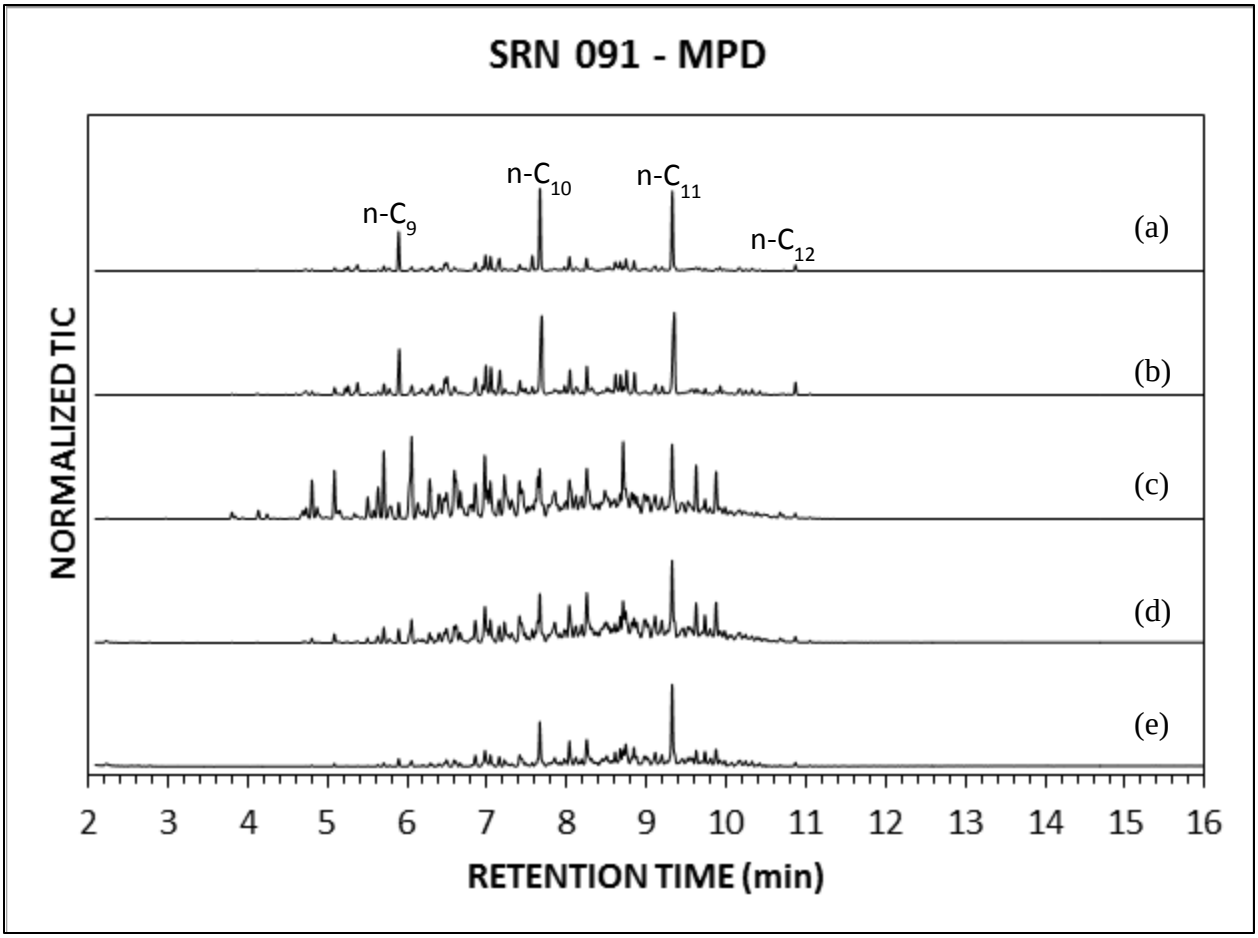

Figure A-20 Microbial degradation of a medium petroleum distillate, SRN091: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

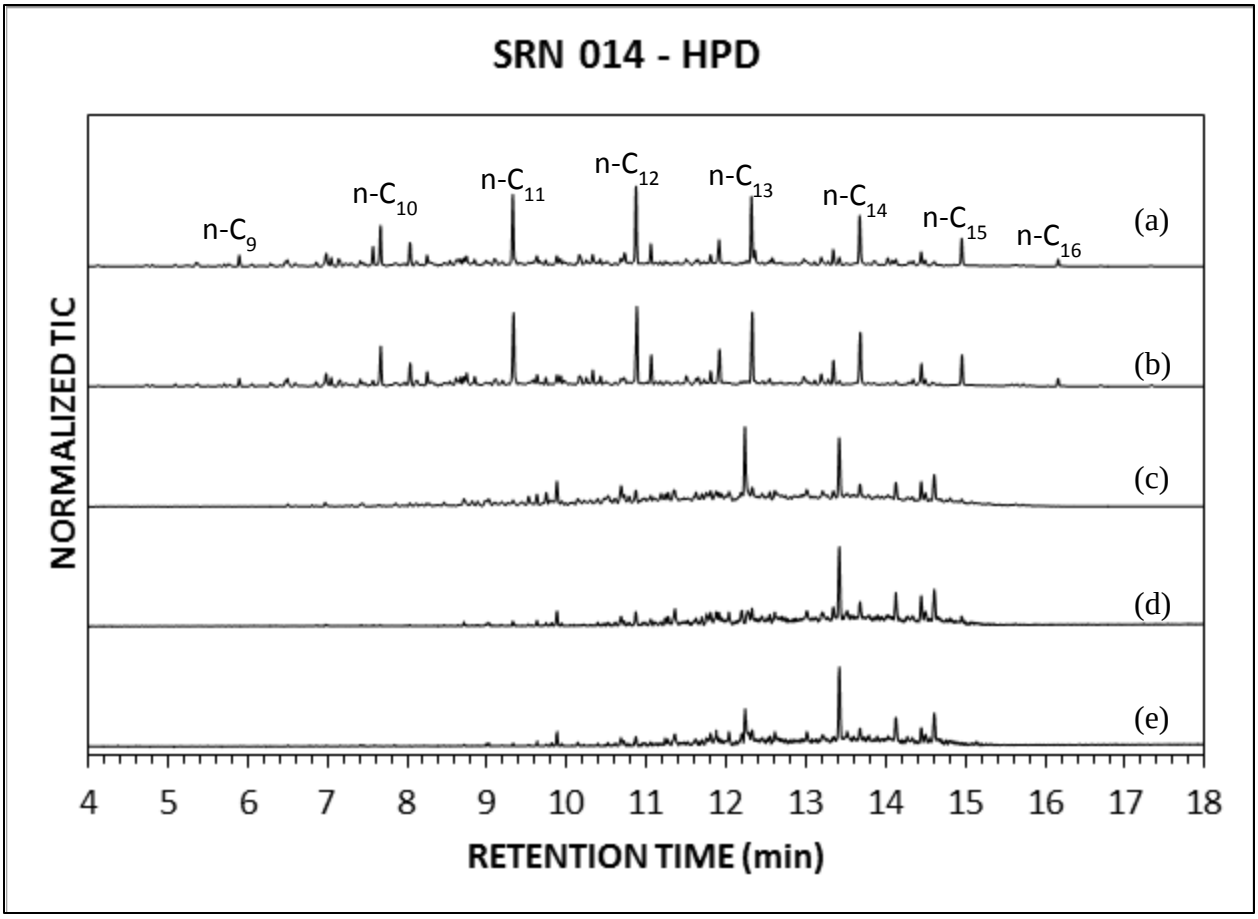

Figure A-21 Microbial degradation of a heavy petroleum distillate, SRN014: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

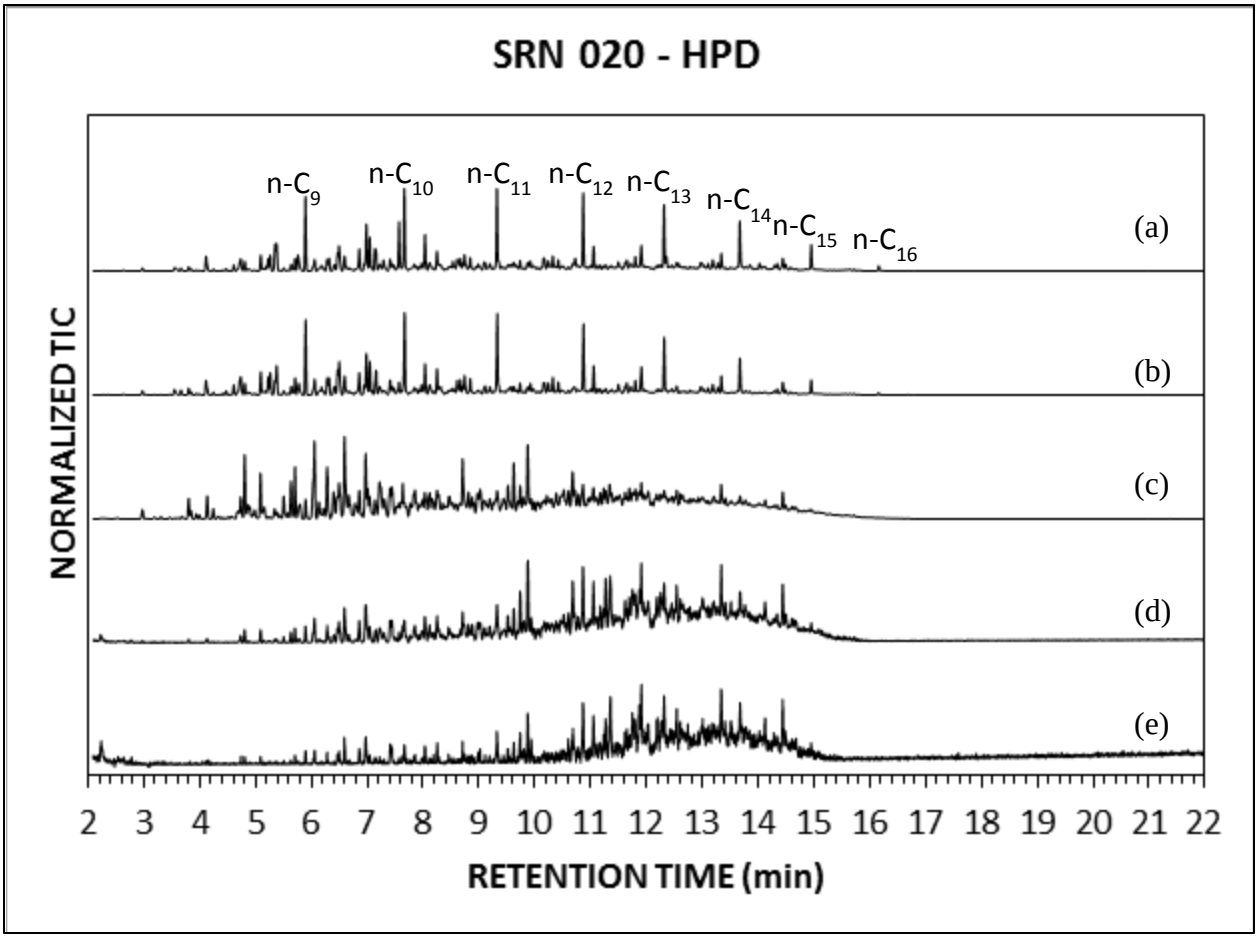

Figure A-22 Microbial degradation of a heavy petroleum distillate, SRN020: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

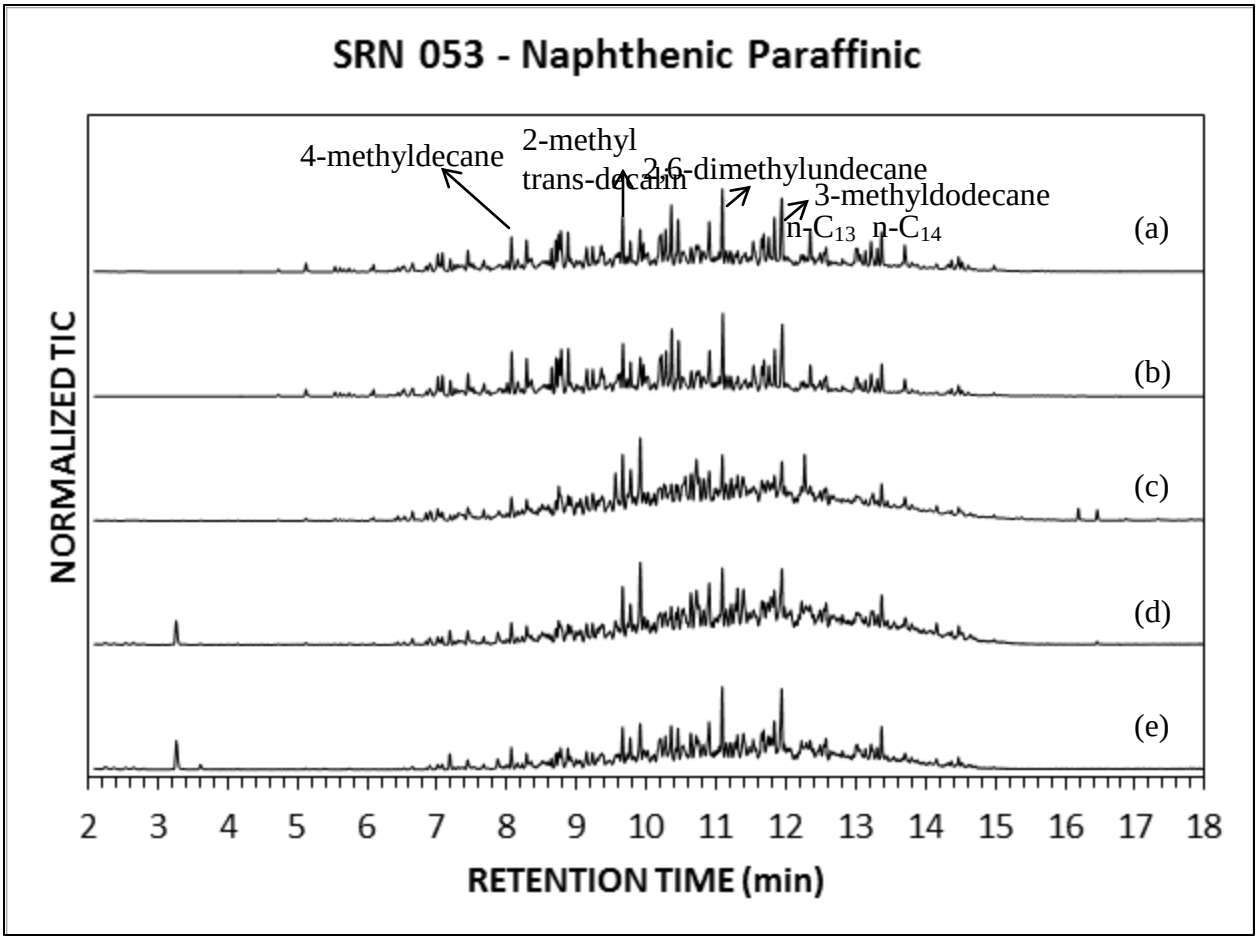

Figure A-23 Microbial degradation of a naphthenic paraffinic product, SRN053: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21.

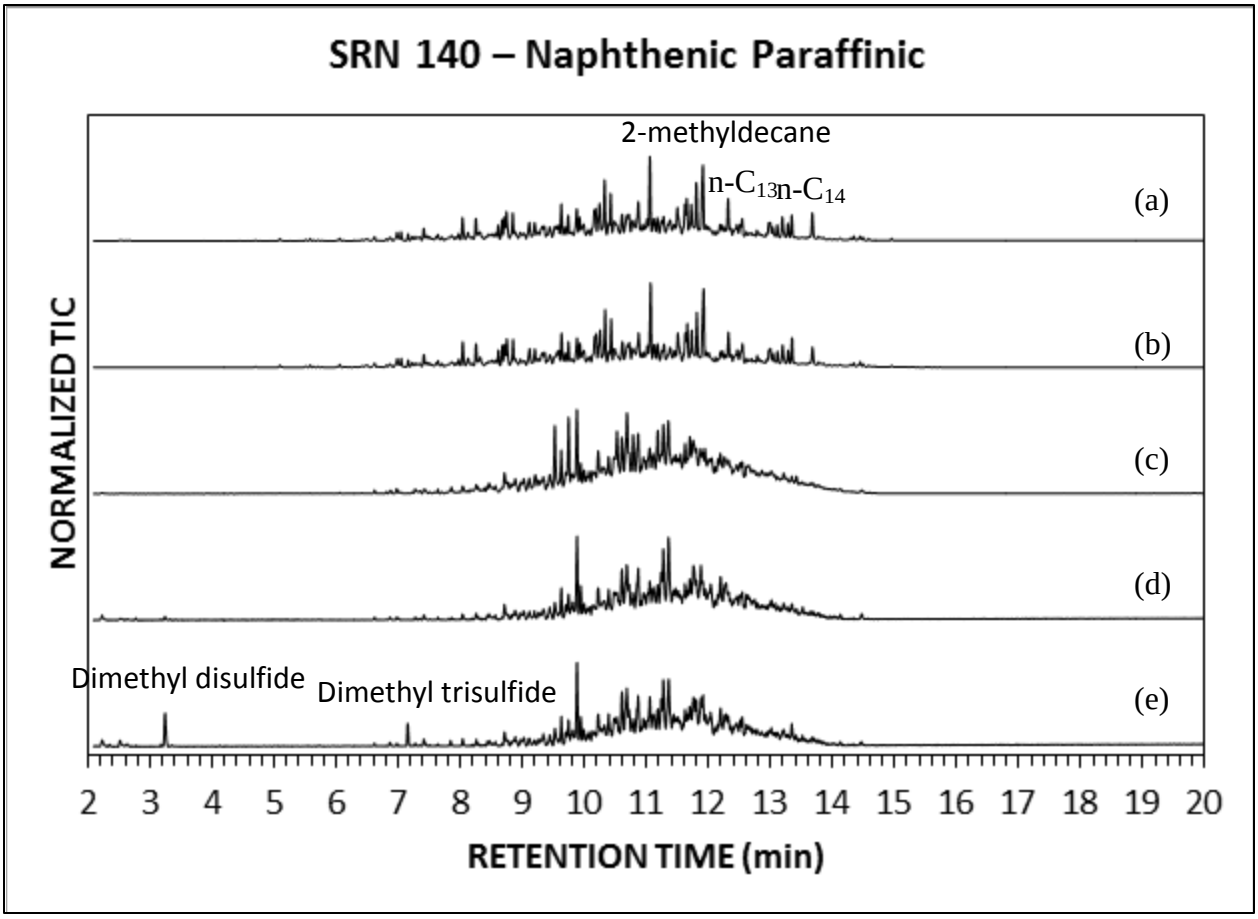

Figure A-24 Microbial degradation of a naphthenic paraffinic product, SRN140: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

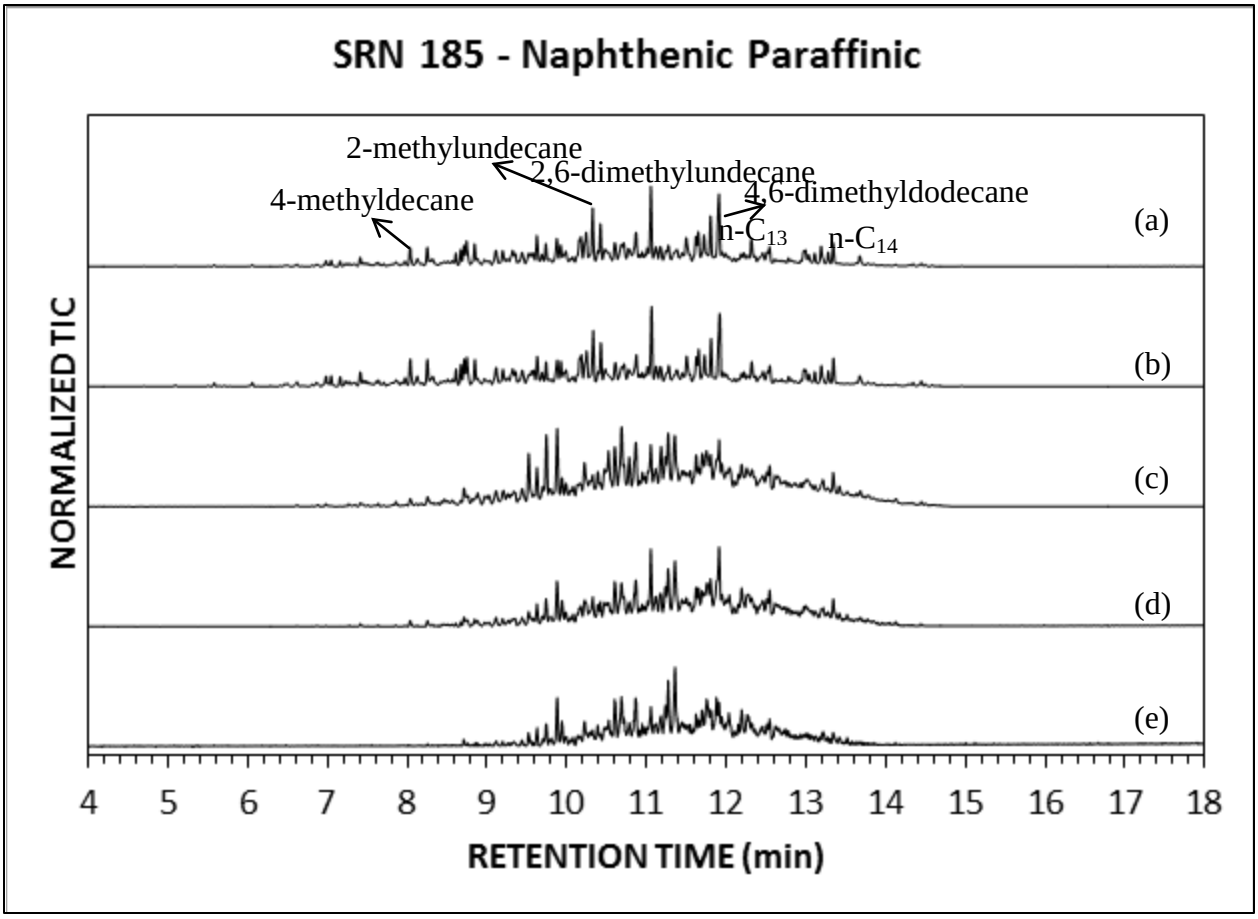

Figure A-25 Microbial degradation of a naphthenic paraffinic product, SRN185: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

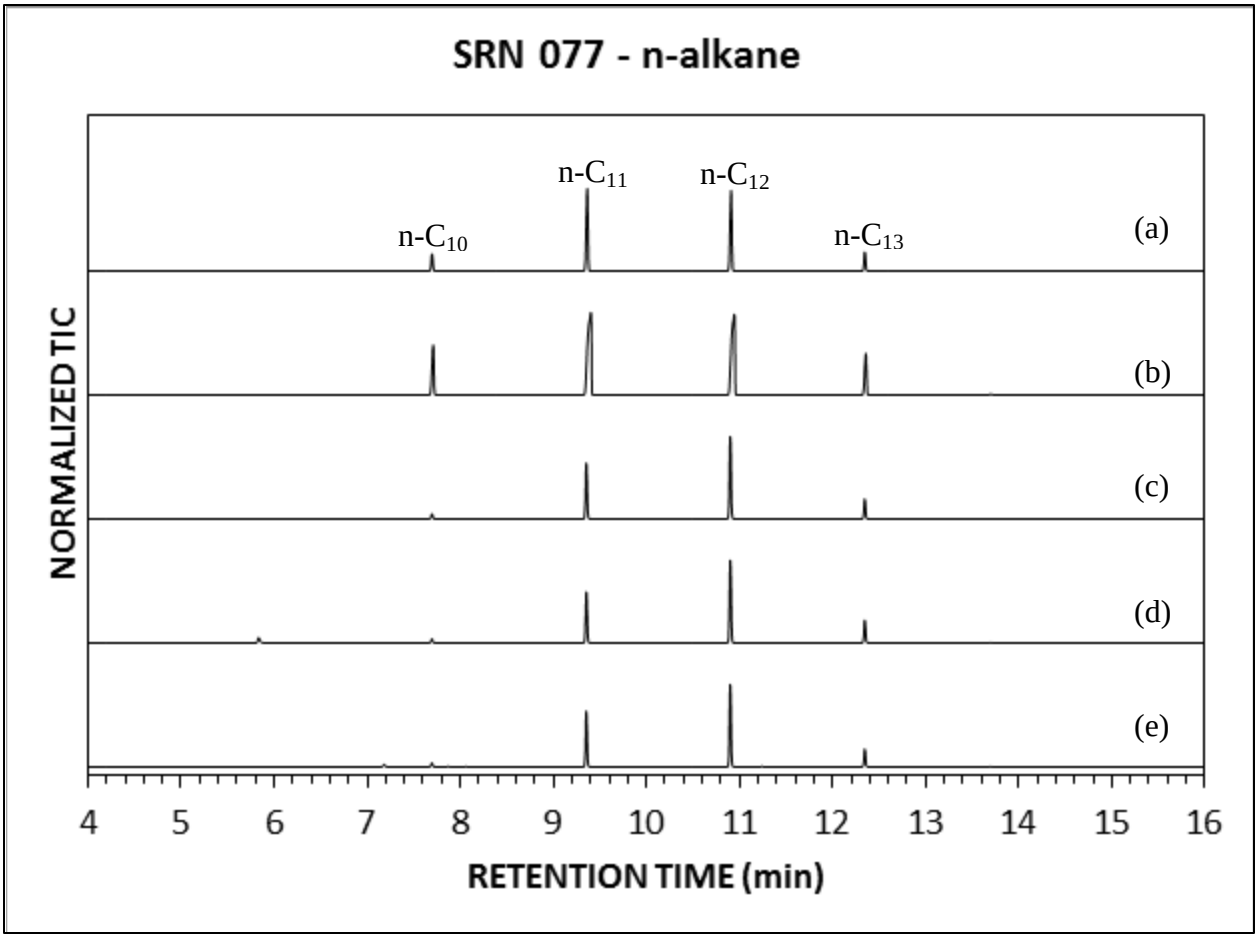

Figure A-26 Microbial degradation of an n-alkane product, SRN077: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21.

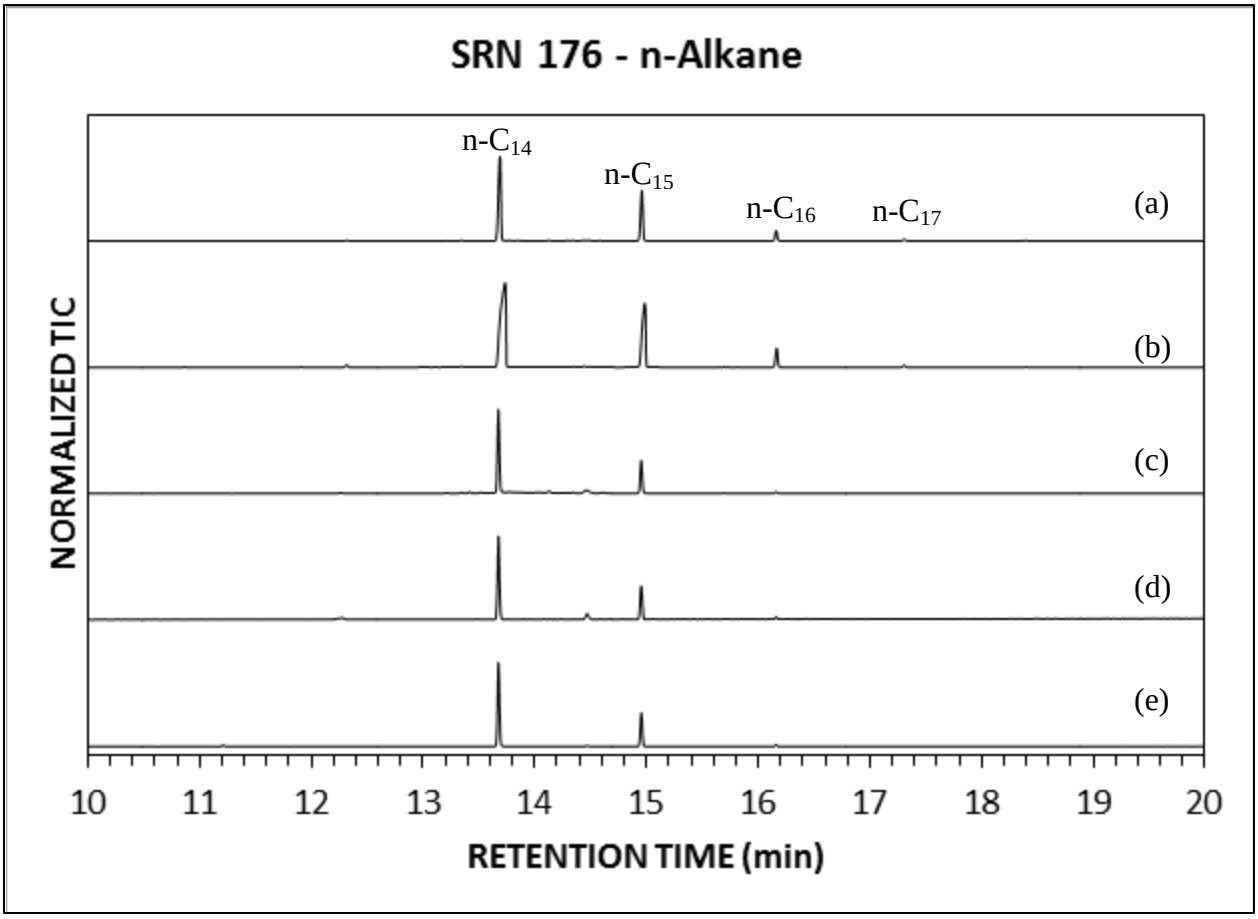

Figure A-27 Microbial degradation of an n-alkane product, SRN176: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

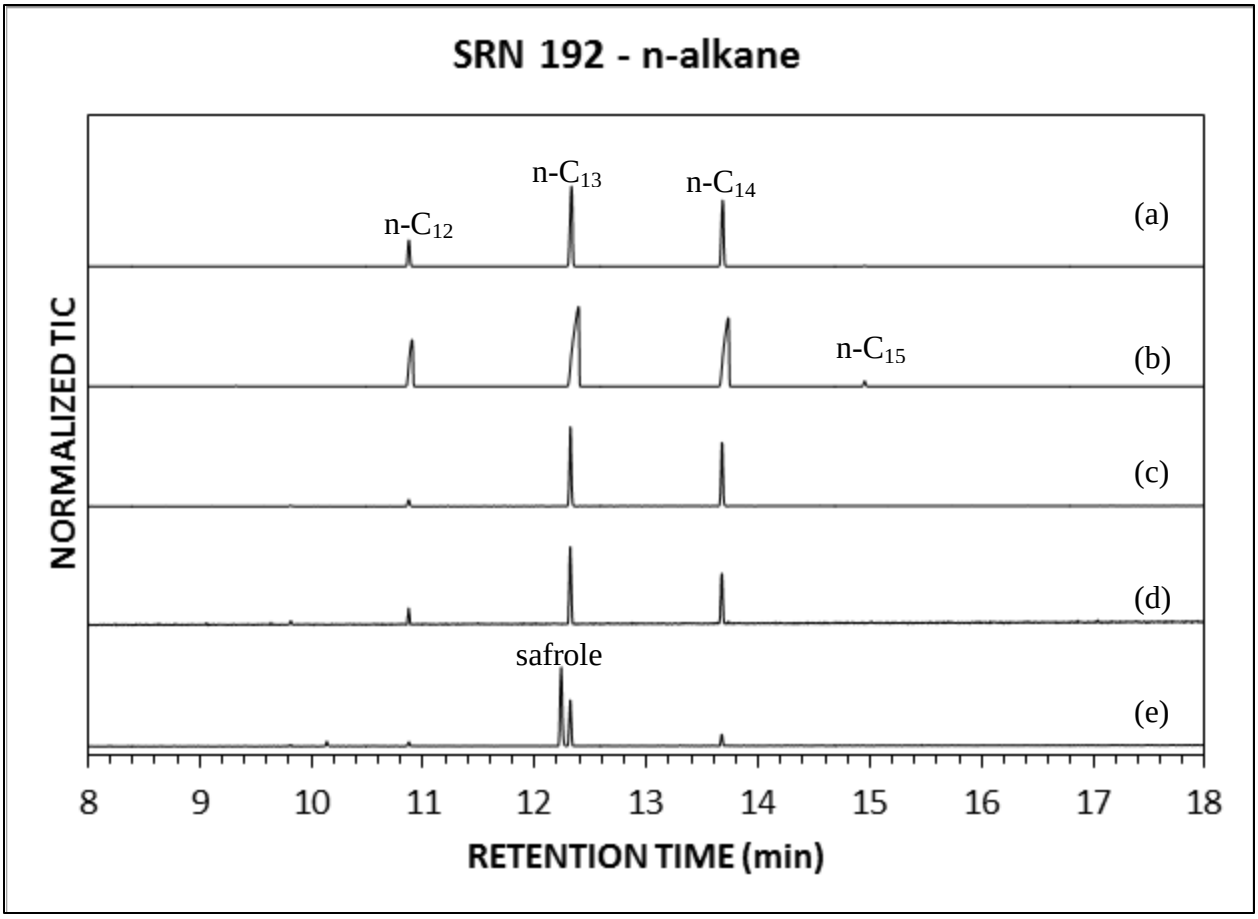

Figure A-28 Microbial degradation of an n-alkane product, SRN192: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

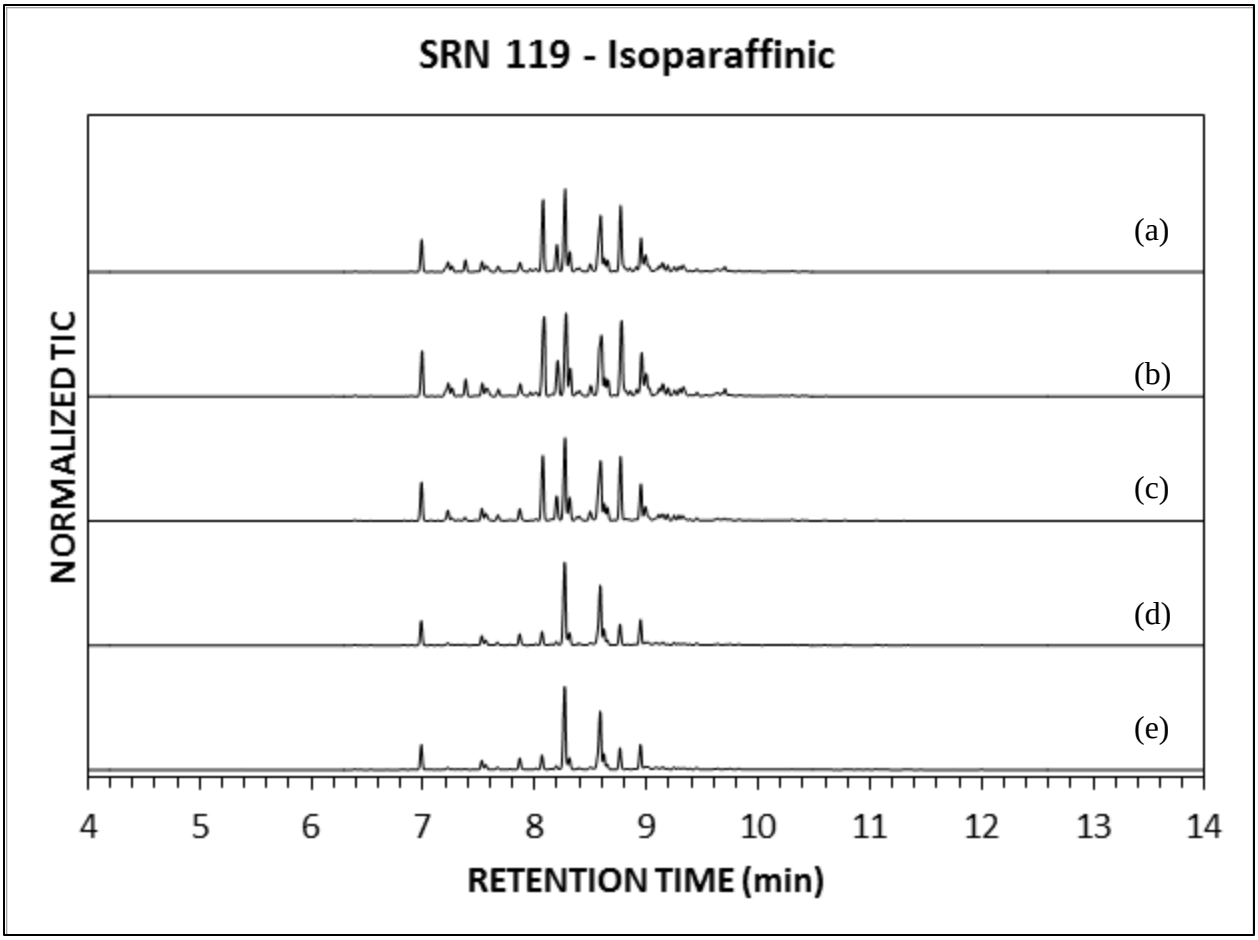

Figure A-29 Microbial degradation of an isoparaffinic product, SRN119: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21. This product contains all branched alkanes for which no standards were obtained and library match scores were low.

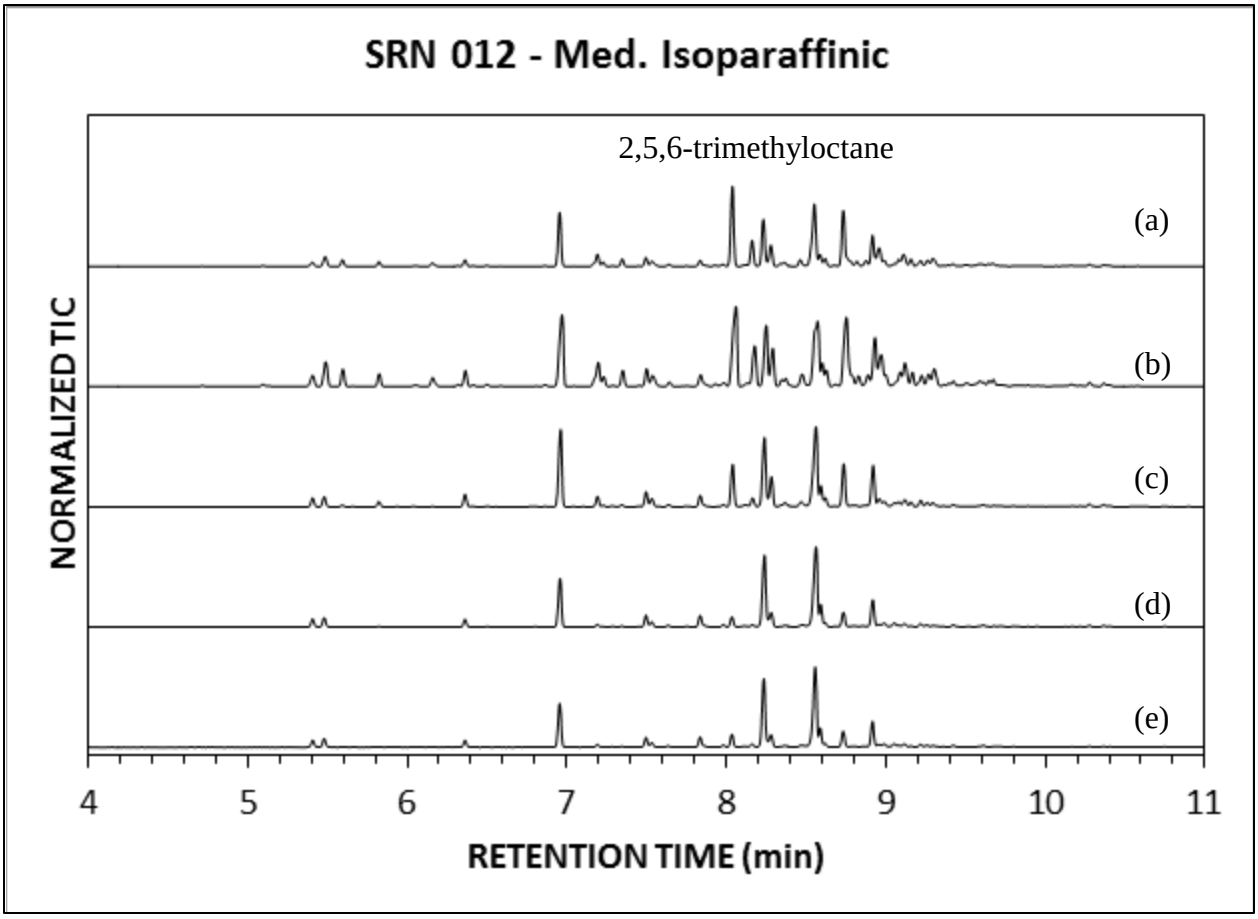

Figure A-30 Microbial degradation of a medium isoparaffinic product, SRN012: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21. Other peaks could not be identified based on poor library match scores.

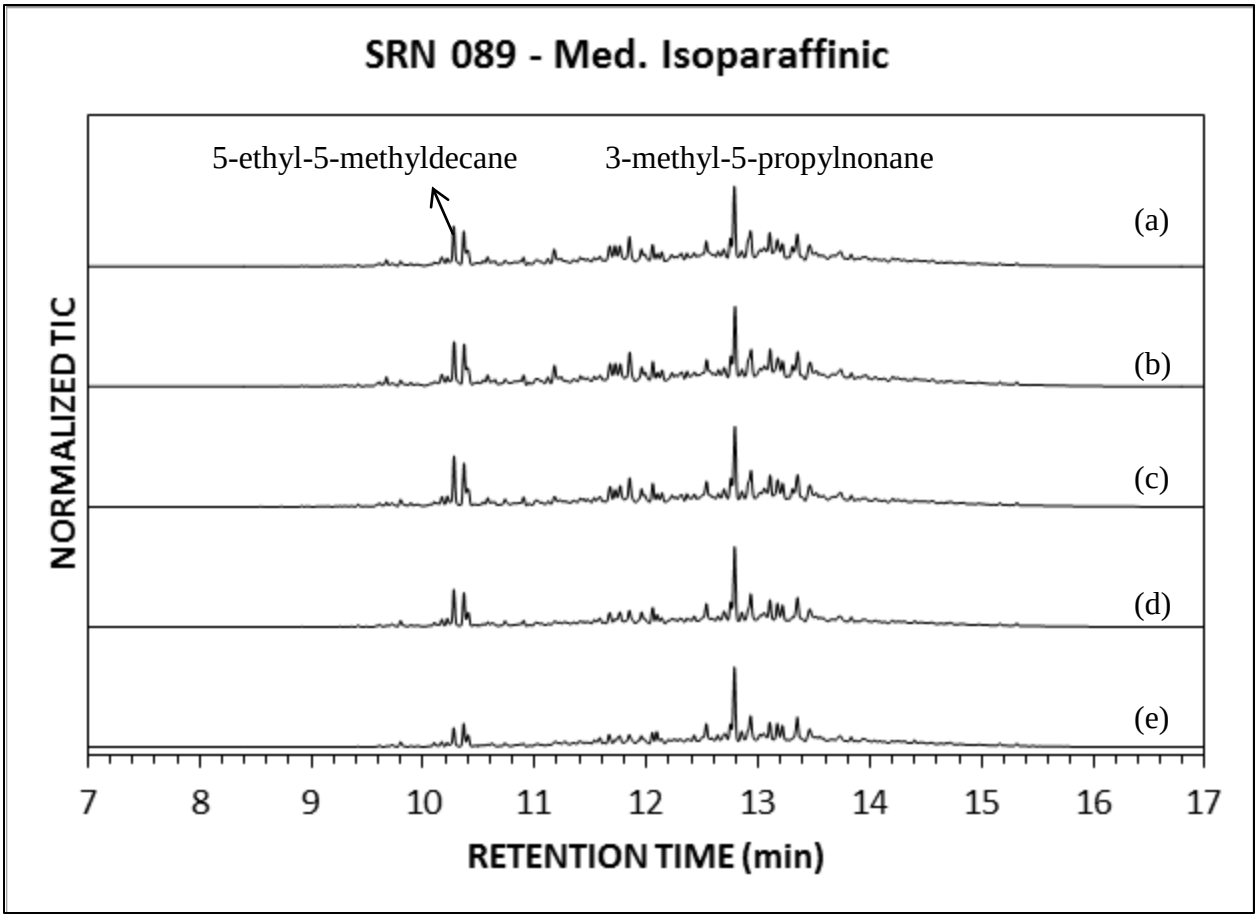

Figure A-31 Microbial degradation of a medium isoparaffinic product, SRN089: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

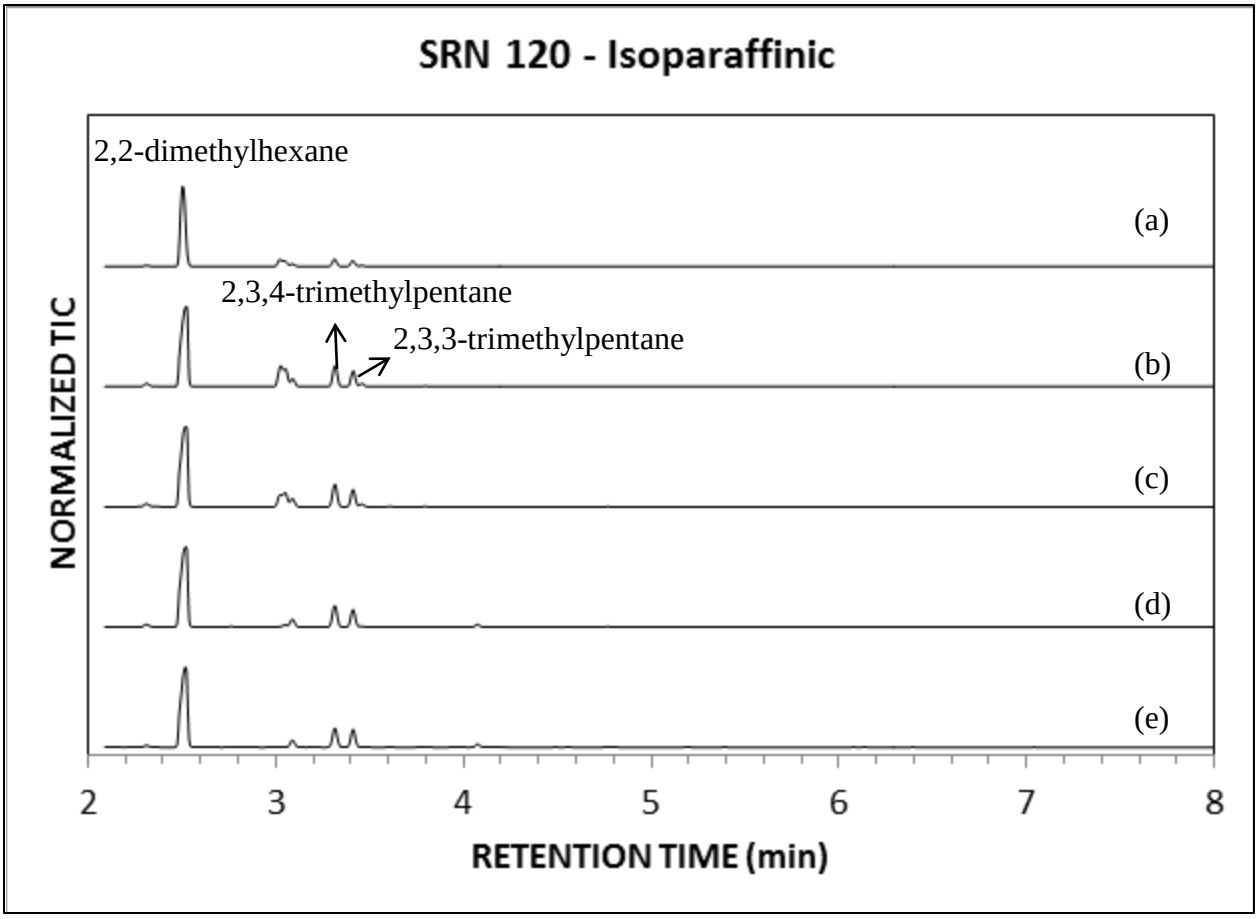

Figure A-32 Microbial degradation of a light isoparaffinic product, SRN120: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

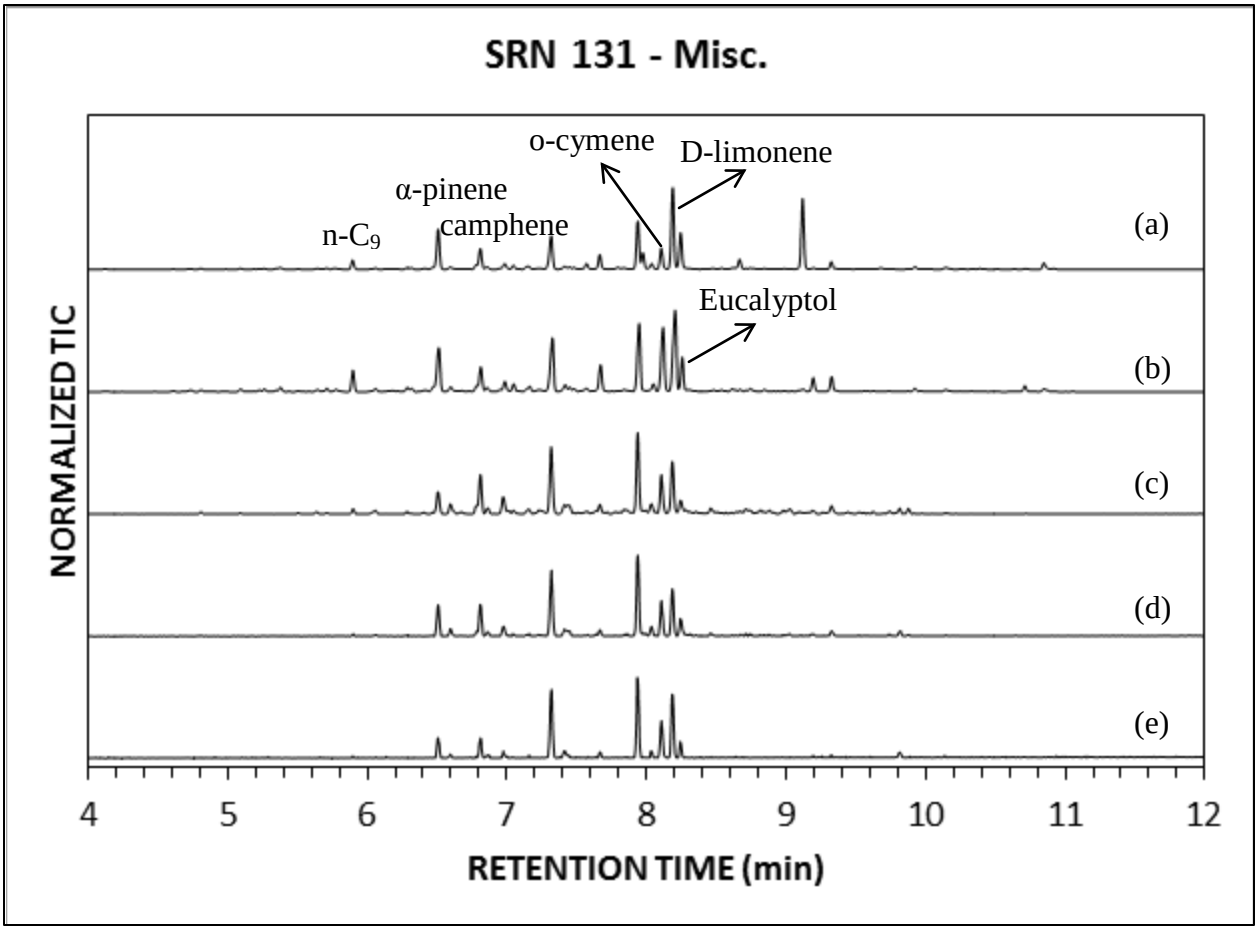

Figure A-33 Microbial degradation of an miscellaneous product, SRN131: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21.

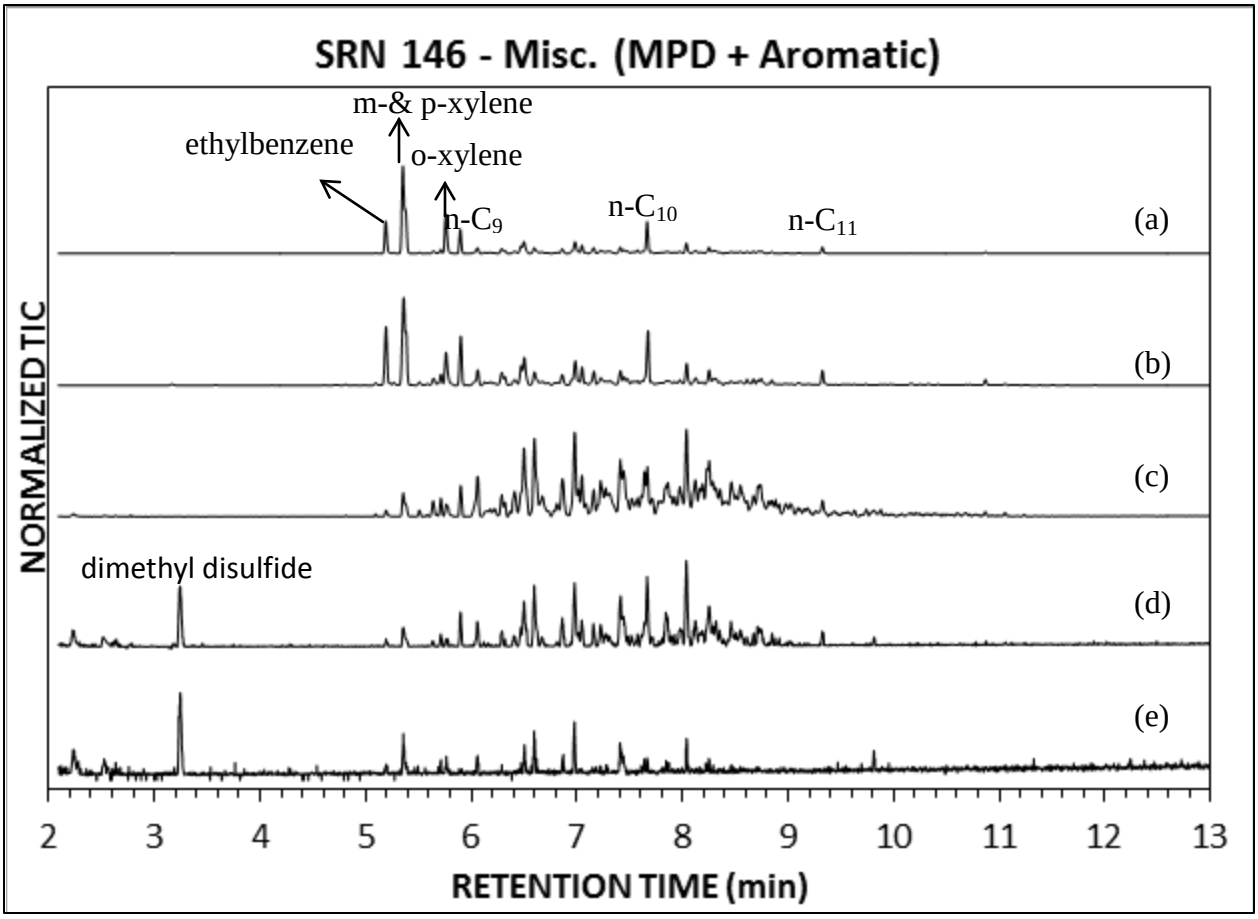

Figure A-34 Microbial degradation of a miscellaneous product (MPD + Aromatic), SRN146: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

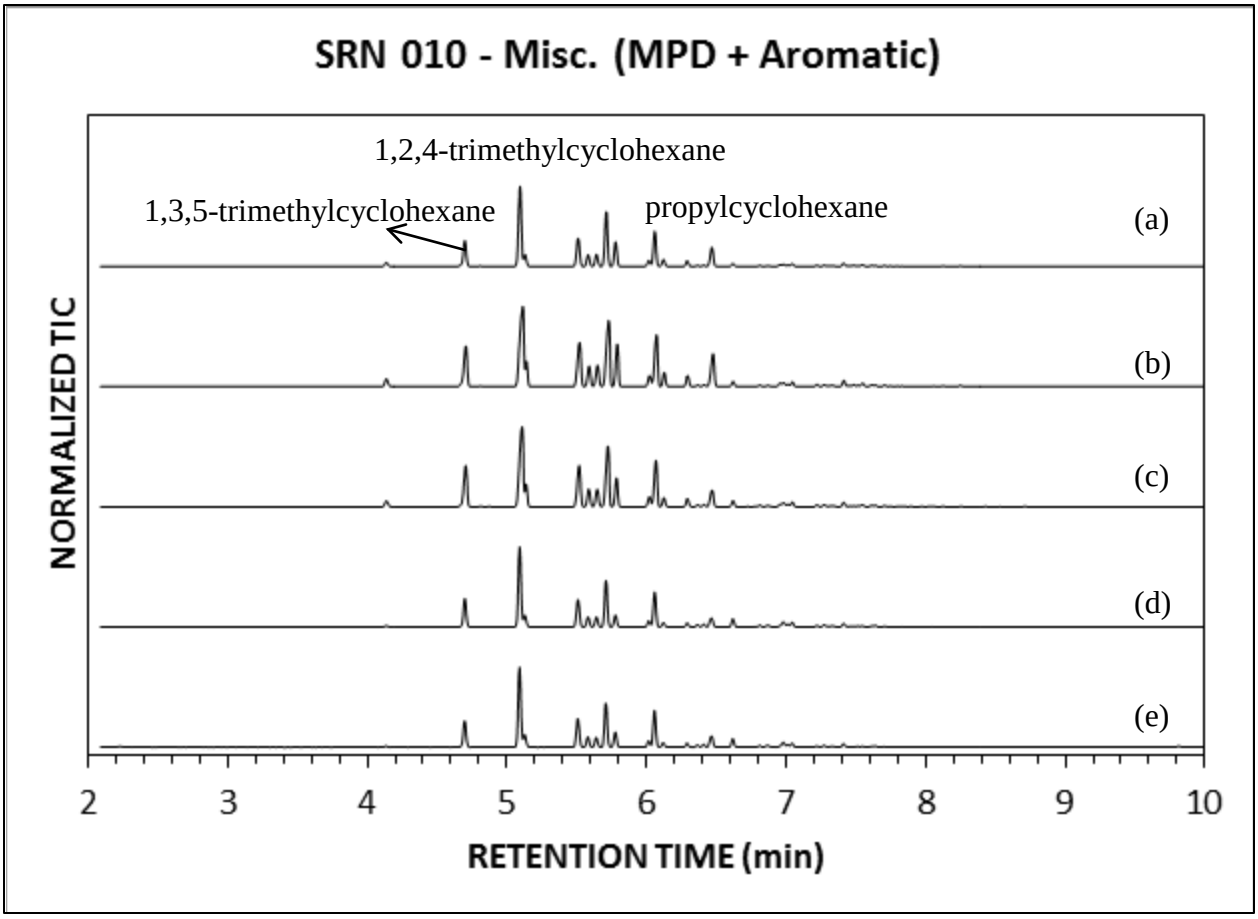

Figure A-35 Microbial degradation of a miscellaneous product (MPD + Aromatic), SRN010: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

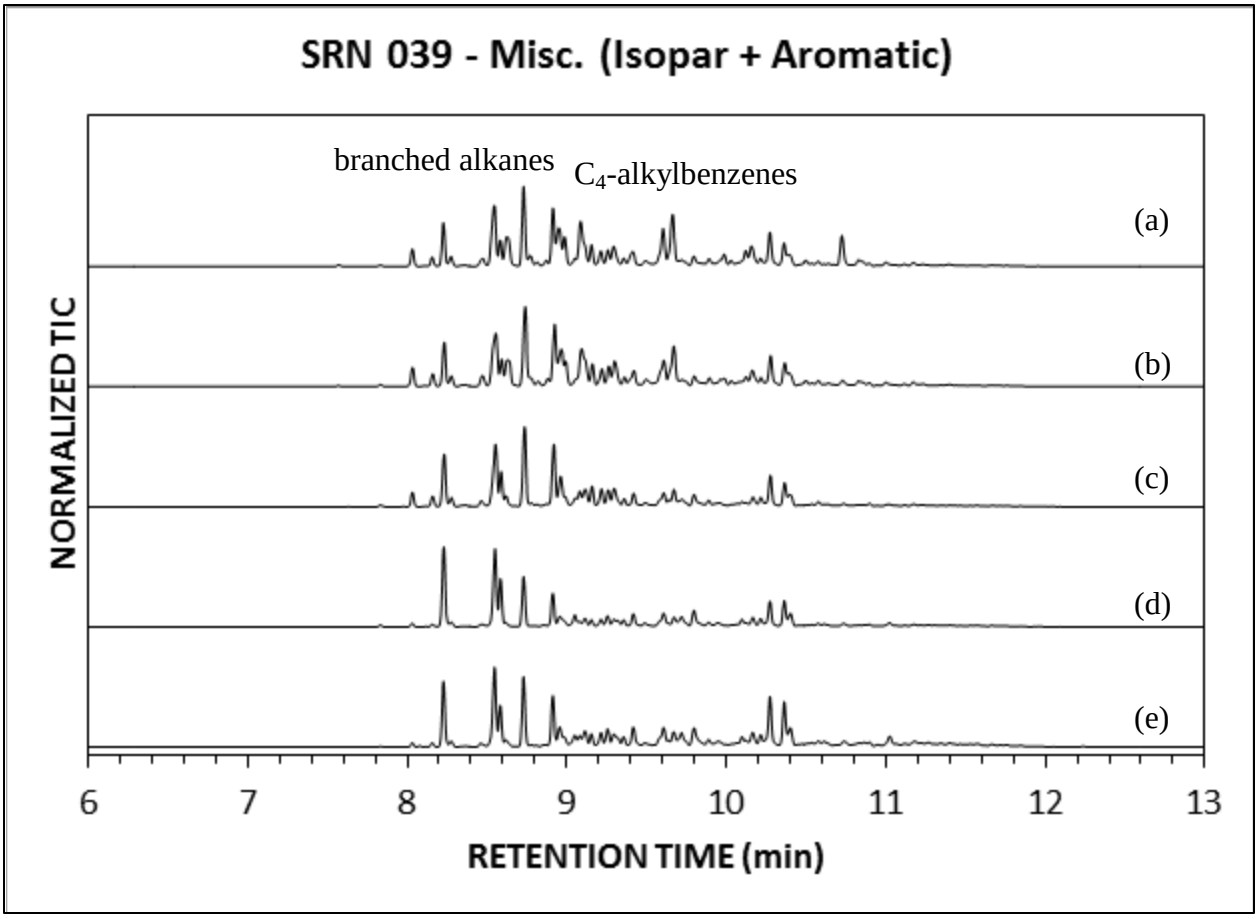

Figure A-36 Microbial degradation of a miscellaneous product (Isoparaffinic + Aromatic), SRN039: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

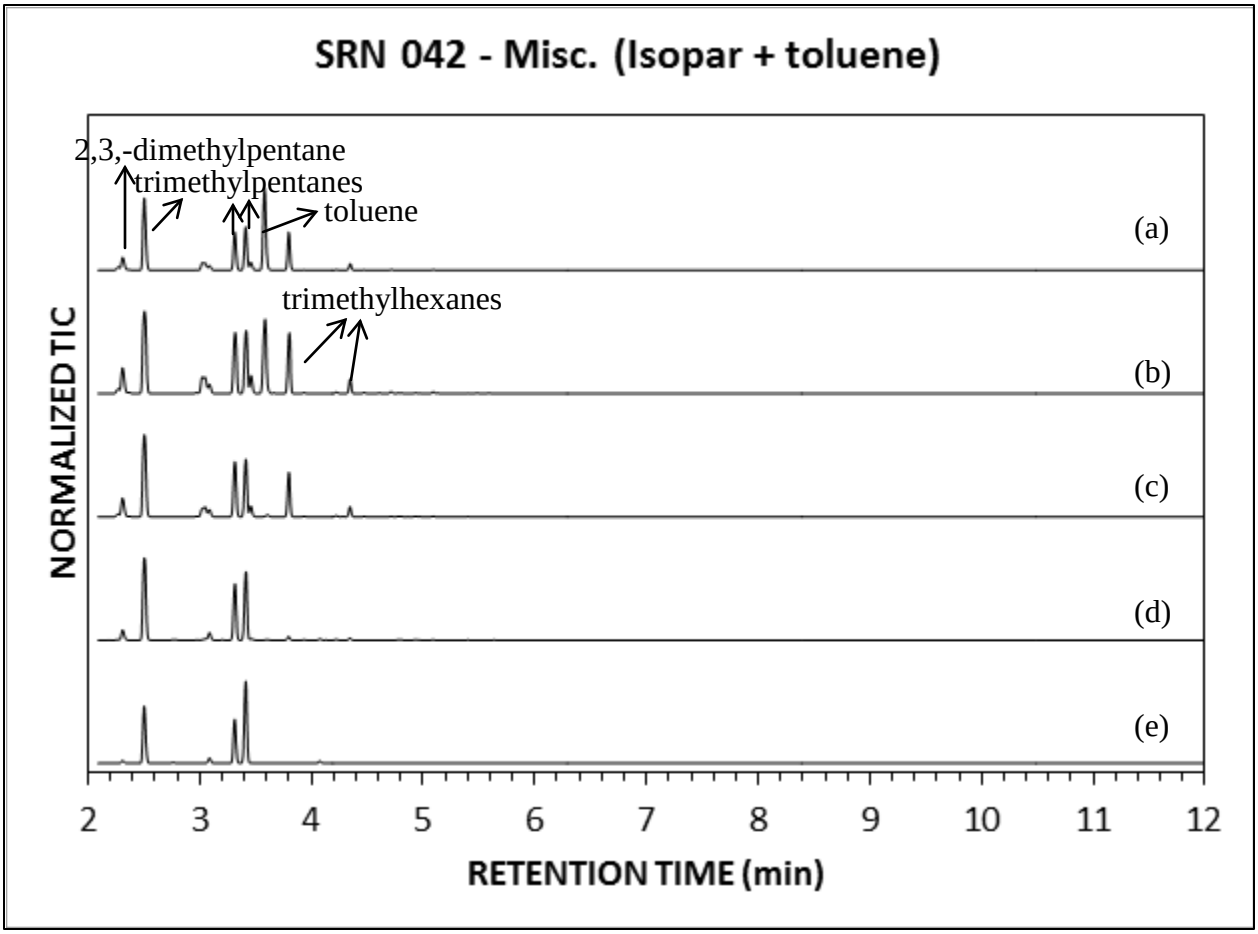

Figure A-37 Microbial degradation of a miscellaneous product (Isoparaffinic + toluene), SRN042: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

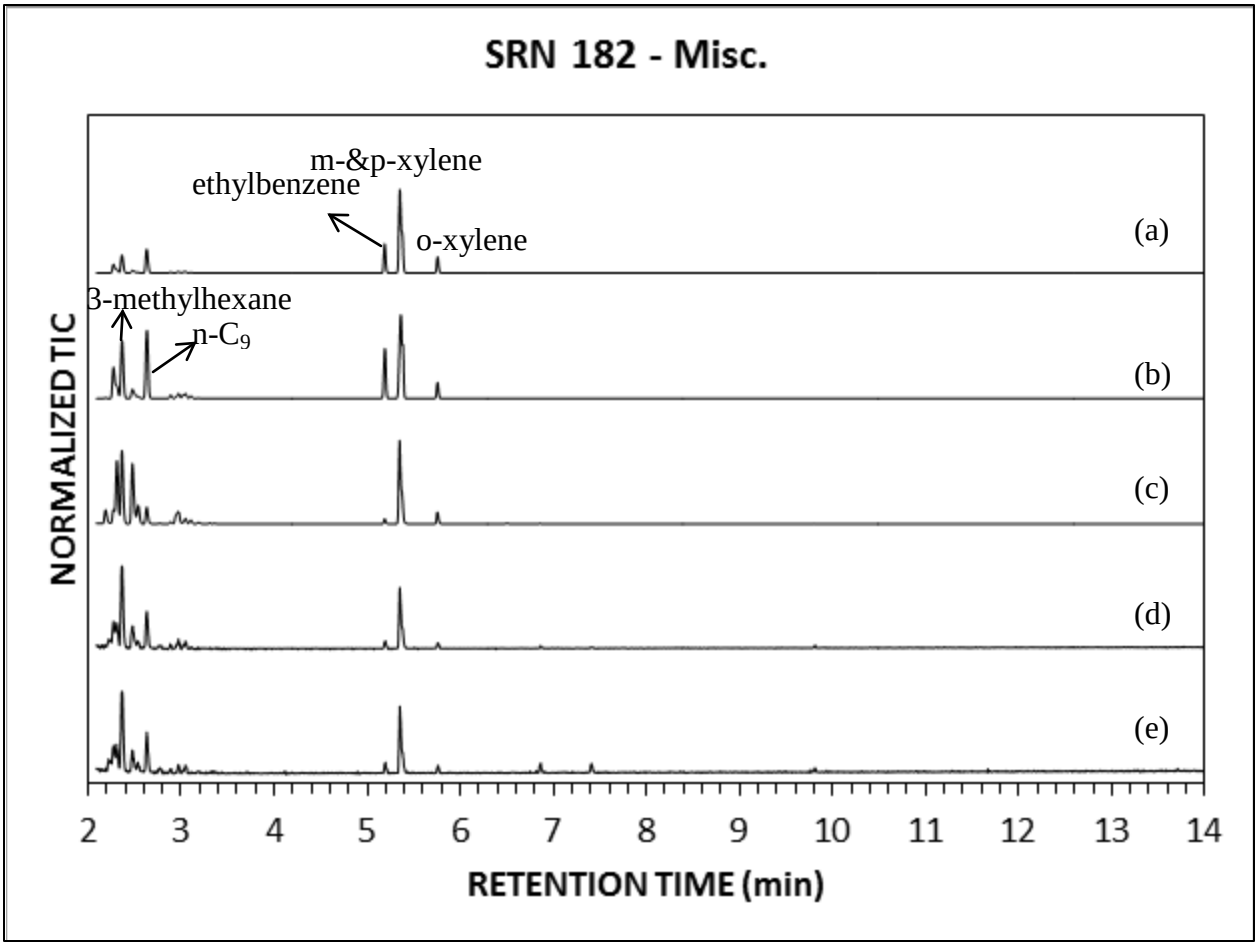

Figure A-38 Microbial degradation of a miscellaneous product, SRN182: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

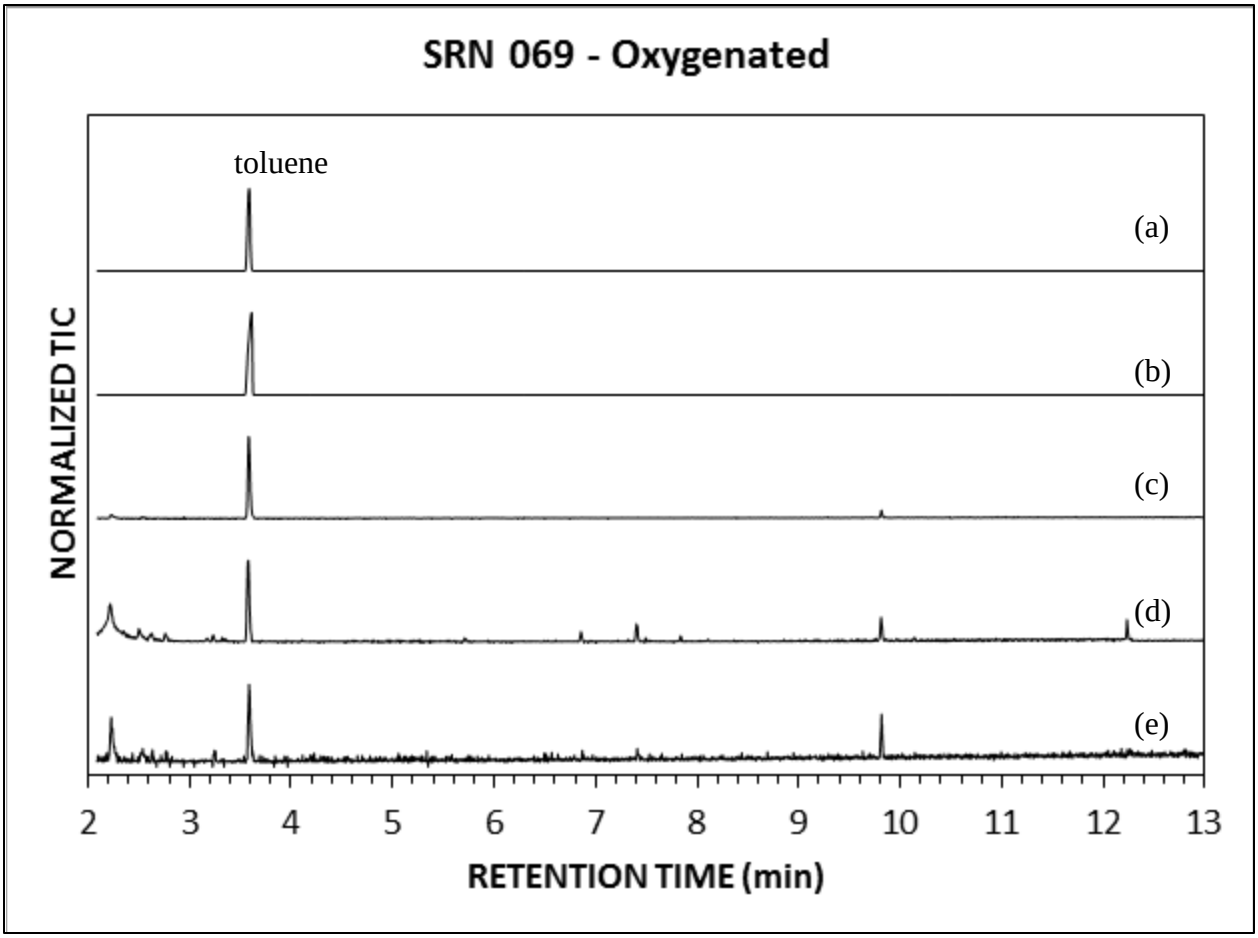

Figure A-39 Microbial degradation of an oxygenated product, SRN069: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21.

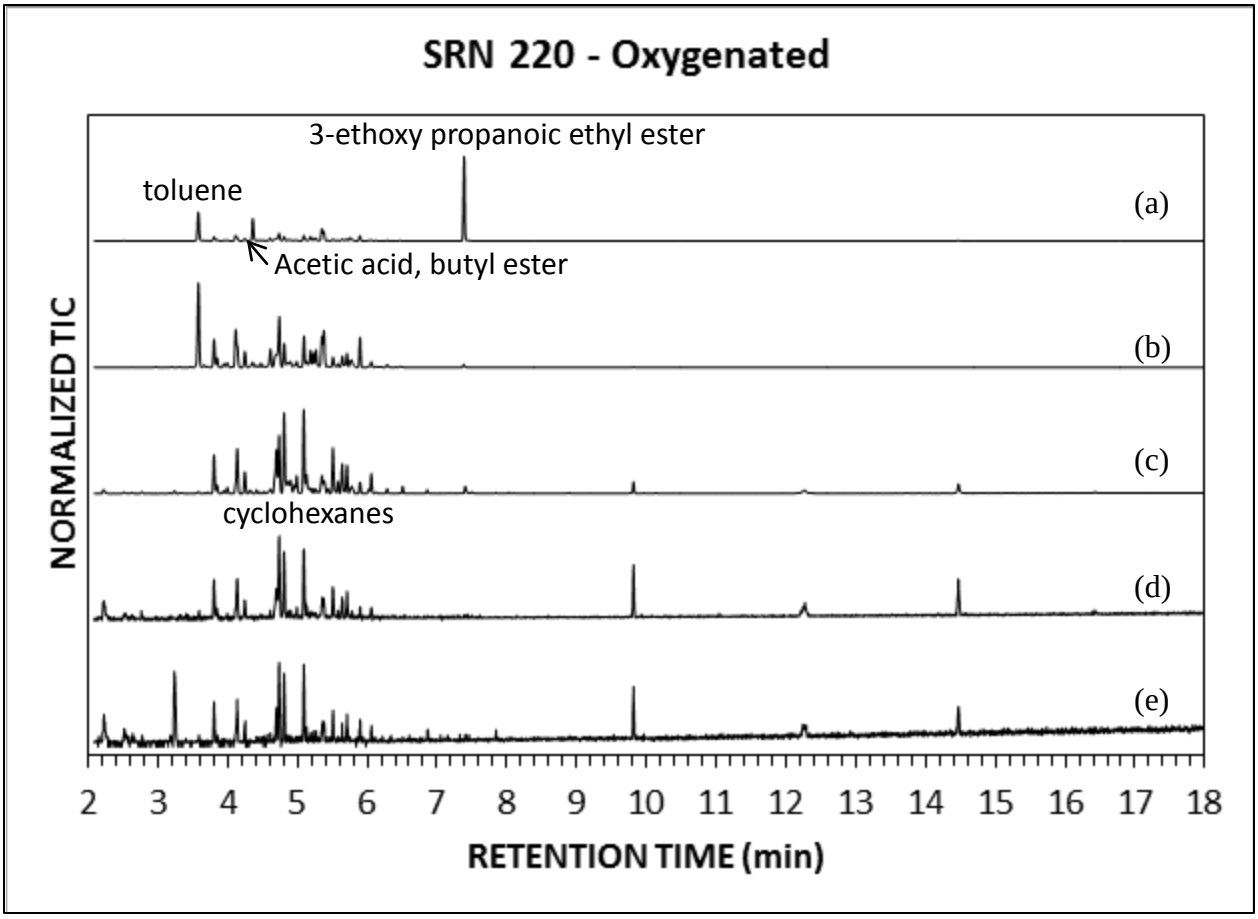

Figure A-40 Microbial degradation of an oxygenated product, SRN220: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

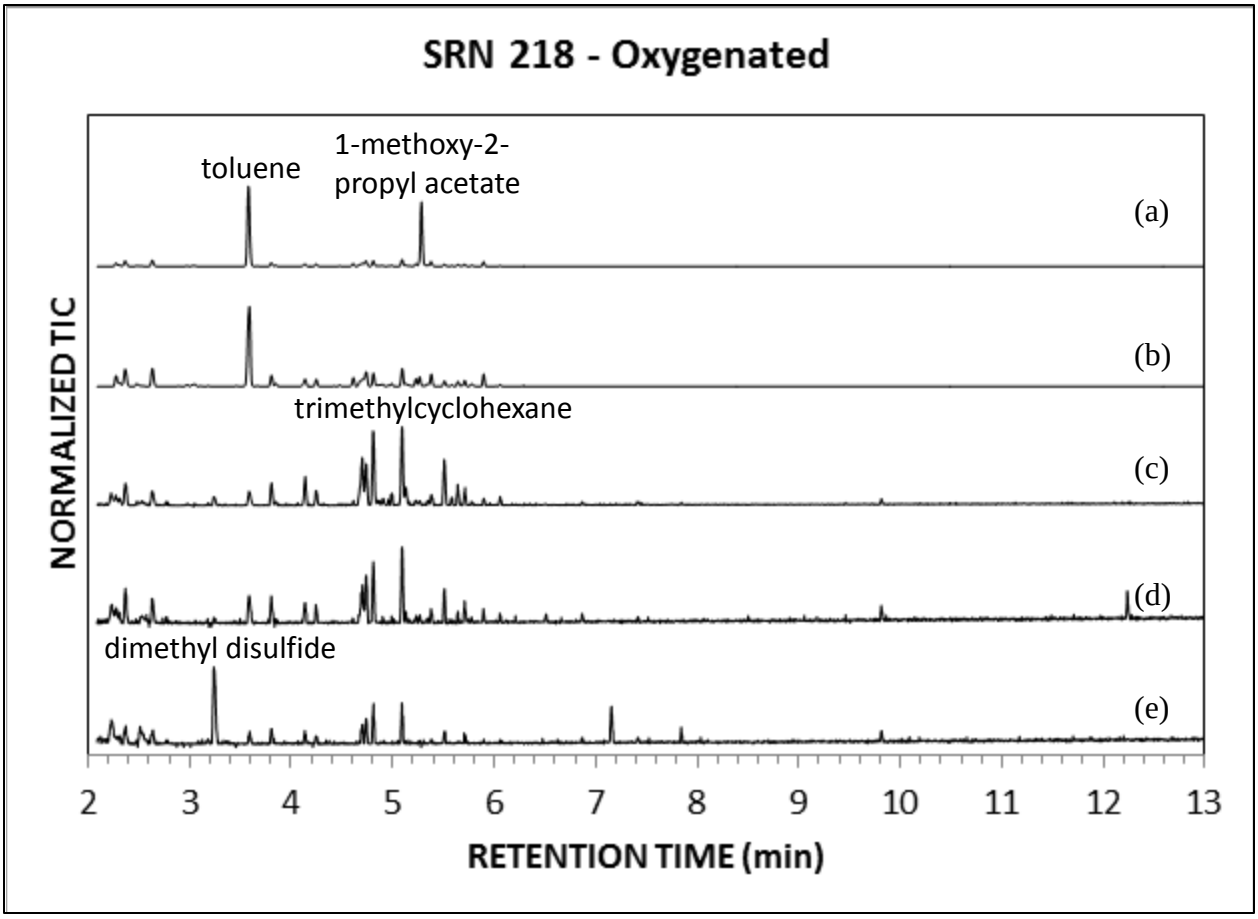

Figure A-41 Microbial degradation of an oxygenated product, SRN218: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

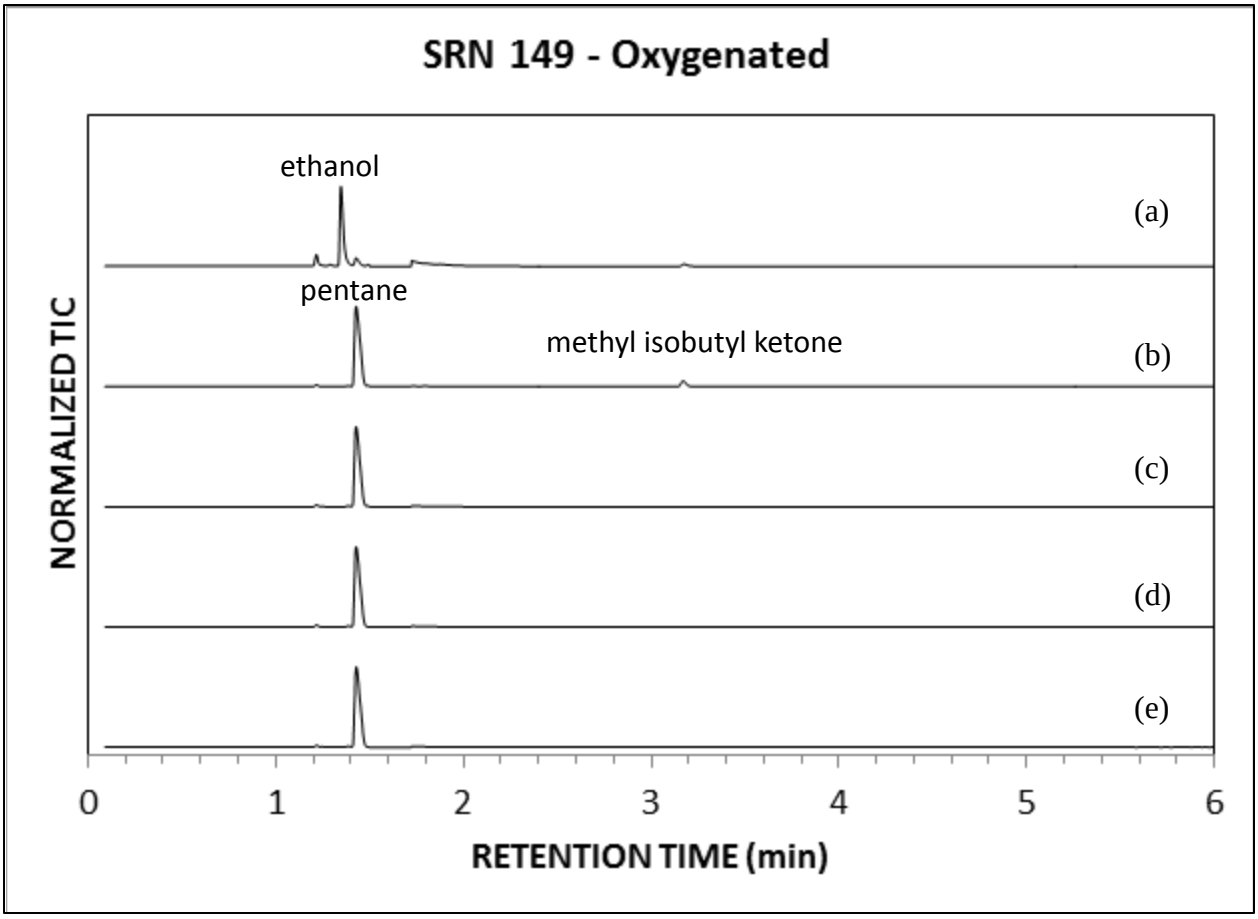

Figure A-42 Microbial degradation of an oxygenated product, SRN149: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, and (e) Day 21.

Appendix B Chromatograms of Oxygenated Liquids Using a Different Headspace Method

Approximately 100g of potting soil was spiked with 20µL of the ignitable liquid. The samples were allowed to age up to 21 days. On the day of analysis a whole carbon strip was suspended into the headspace of the can on a prebaked paper clip using a nylon string. The cans were baked at 85°C for 4h. Upon cooling, the strips were cut in half. One half of the strip was stored and the other half was extracted with 600µL of CS_2. All data was acquired using an Agilent 6890 Gas Chromatograph with an Agilent 5975 Mass Spectrometer. The GC was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 microns). The carrier gas was helium with a flow rate of 1mL/min. The method utilized an inlet temperature of 250°C, 1 µL injection volume, and a 20:1 split ratio. The default oven temperature program started at 40°C for 2 minutes, ramped to 280°C at 10°C/min. and held for 3 minutes. The MS parameters included a timed event to turn the detector off at 1.50 minutes and back on at 1.70 minutes. Additionally, the scan range was 24-300m/z initially and then 33-400m/z after the detector was turned back on at 1.70 minutes.

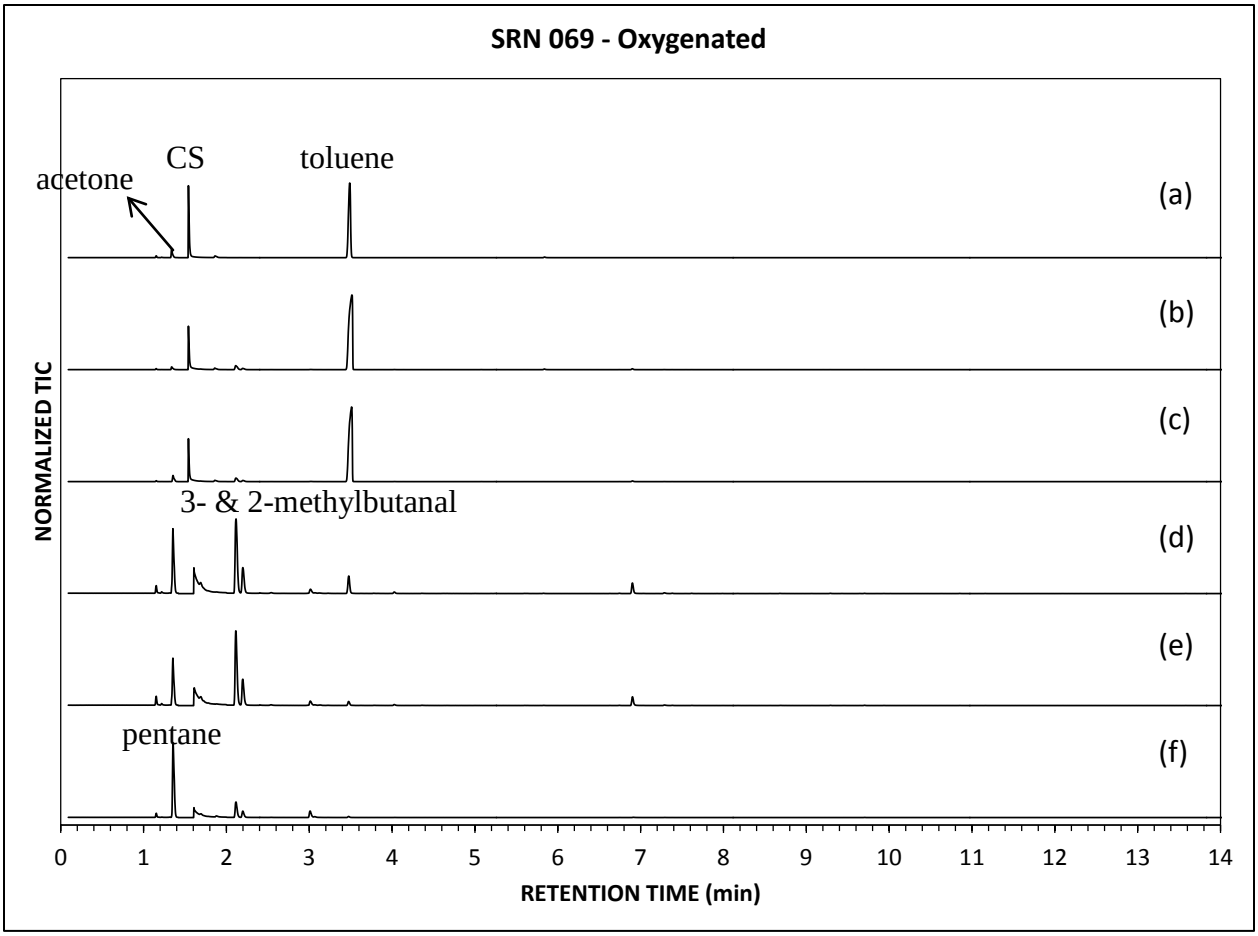

Figure B-1 Microbial degradation of an oxygenated product, SRN069: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 2, (d) Day 7, (e) Day 14, and (f) Day 21.

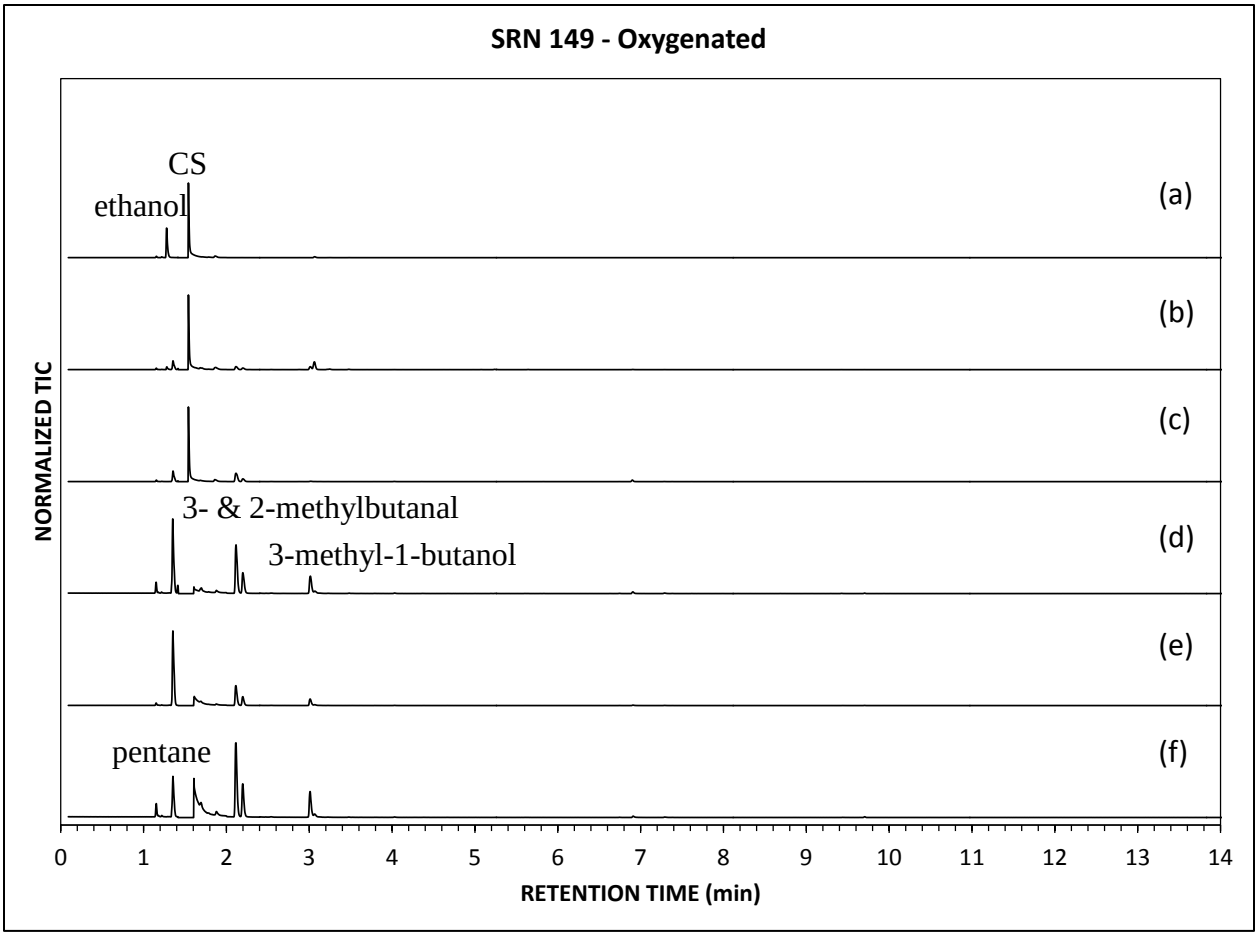

Figure B-2 Microbial degradation of an oxygenated product, SRN149: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 2, (d) Day 7, (e) Day 14, and (f) Day 21.

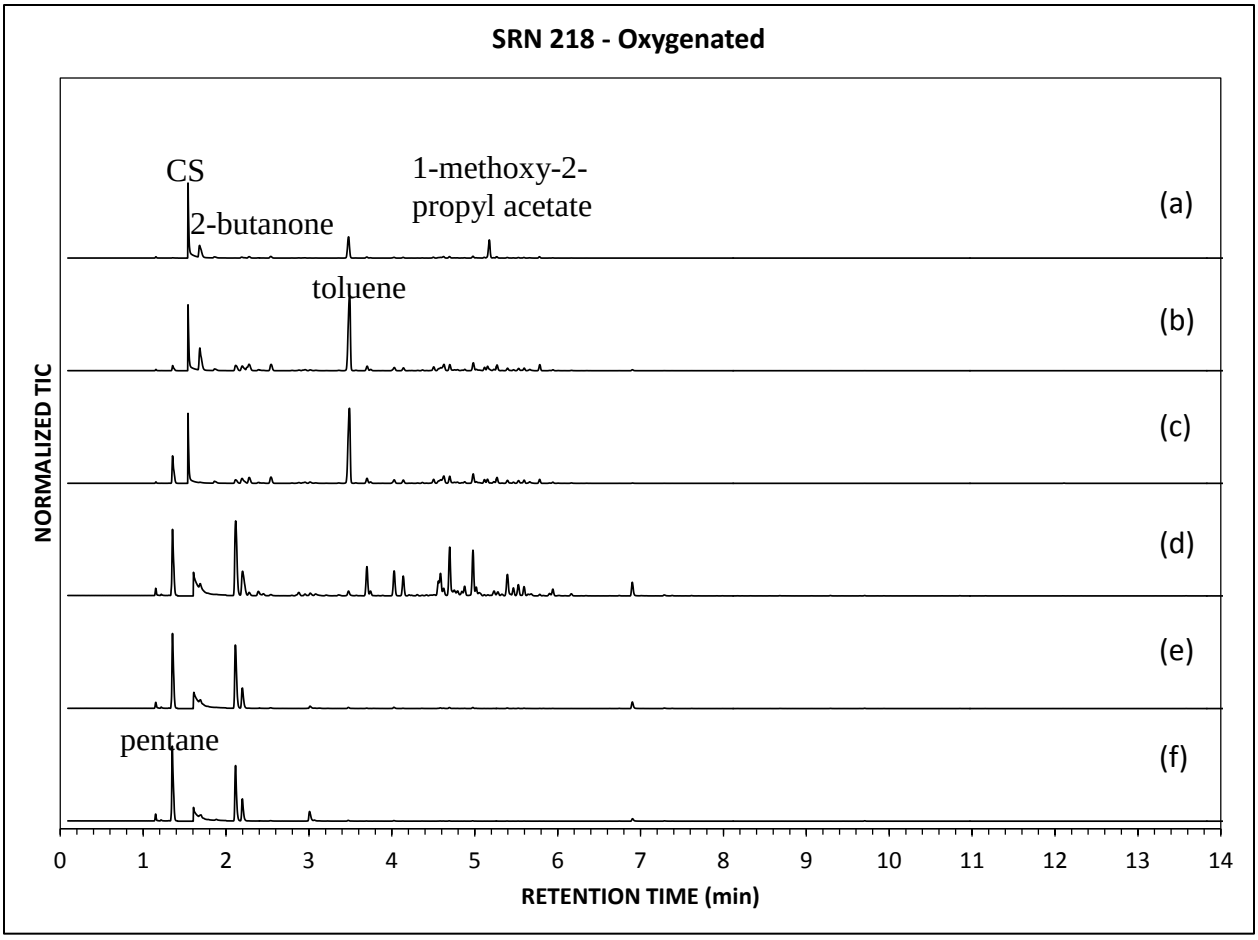

Figure B-3 Microbial degradation of an oxygenated product, SRN218: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 2, (d) Day 7, (e) Day 14, and (f) Day 21.

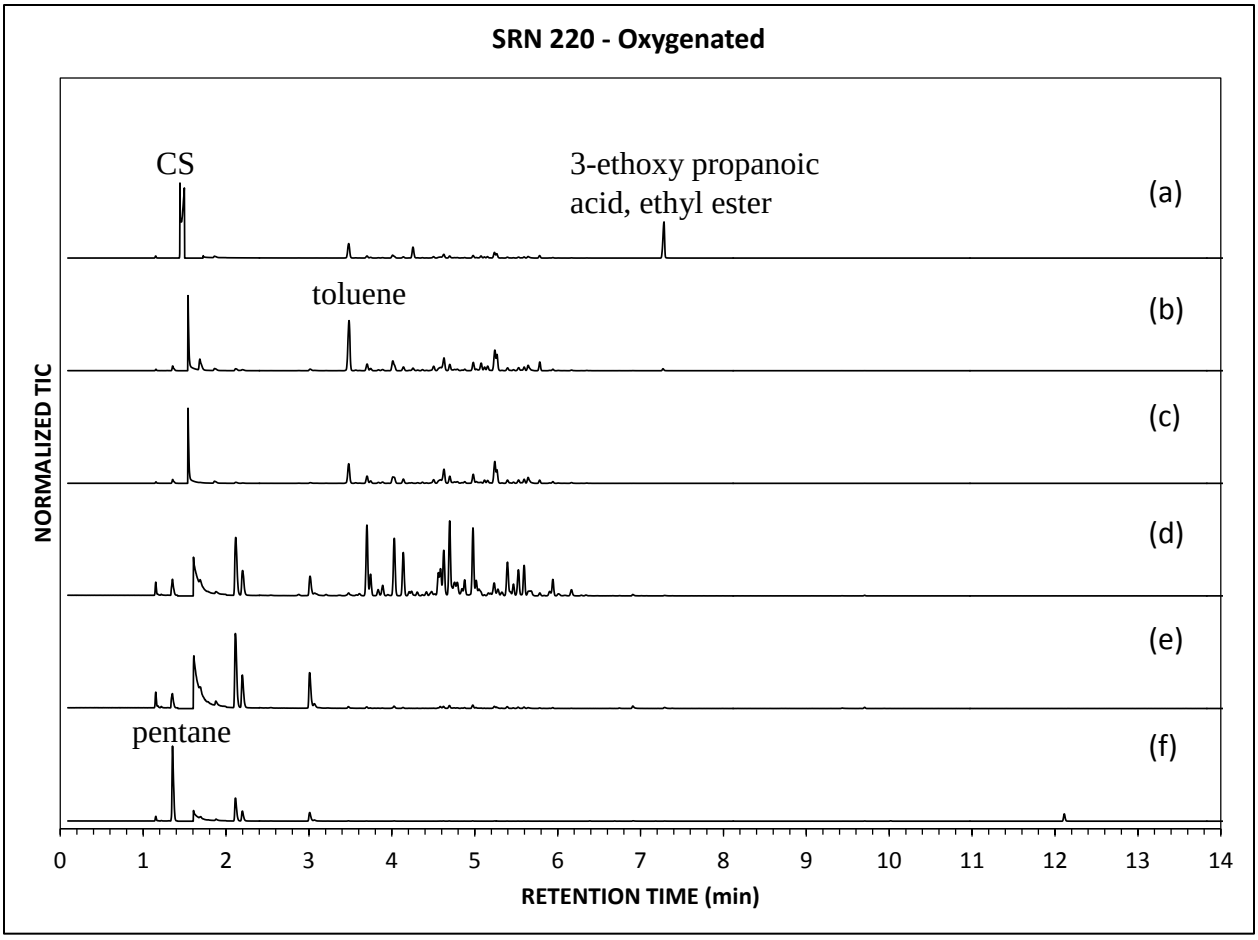

Figure B-4 Microbial degradation of an oxygenated product, SRN220: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 2, (d) Day 7, (e) Day 14, and (f) Day 21.

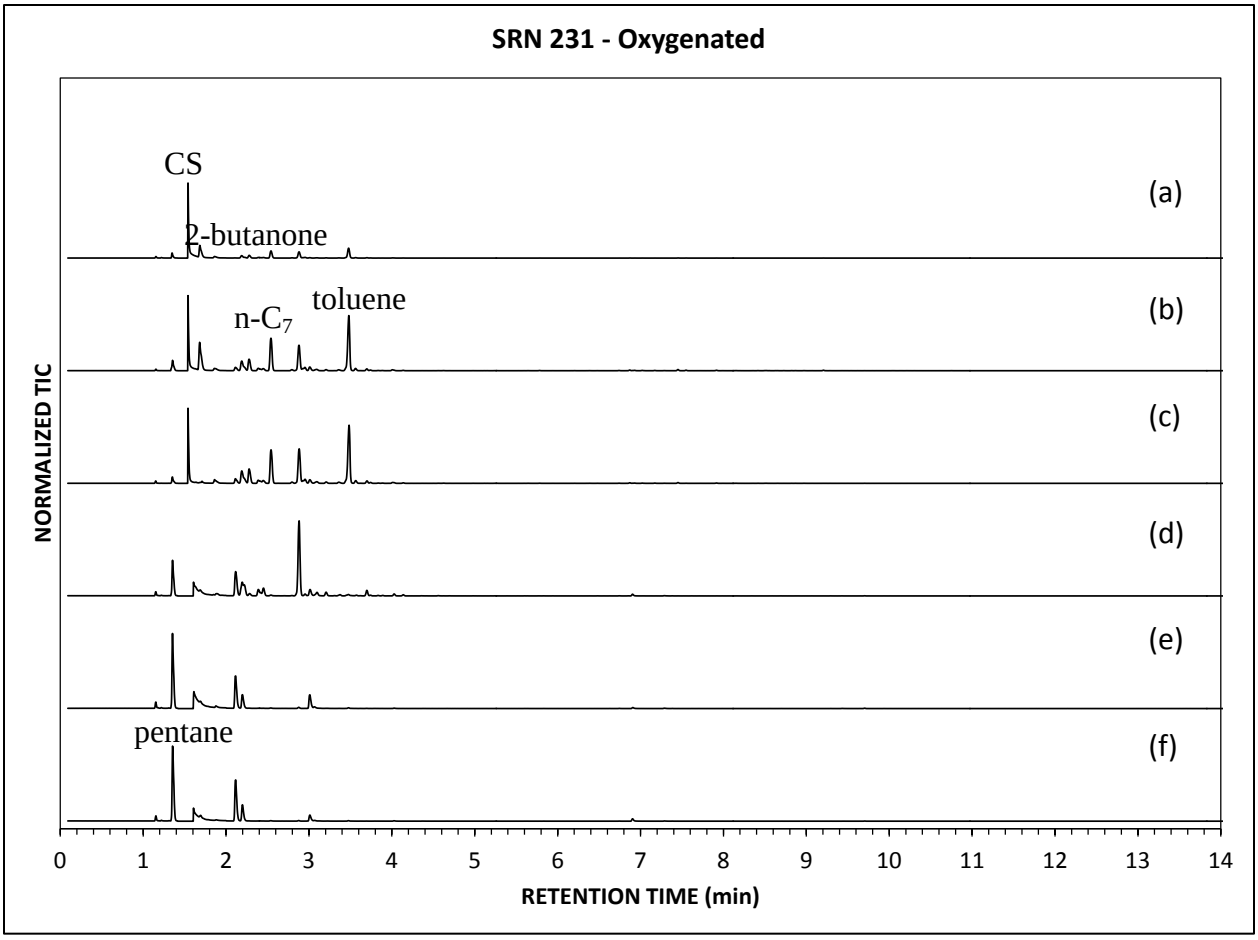

Figure B-5 Microbial degradation of an oxygenated product, SRN231: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 2, (d) Day 7, (e) Day 14, and (f) Day 21.

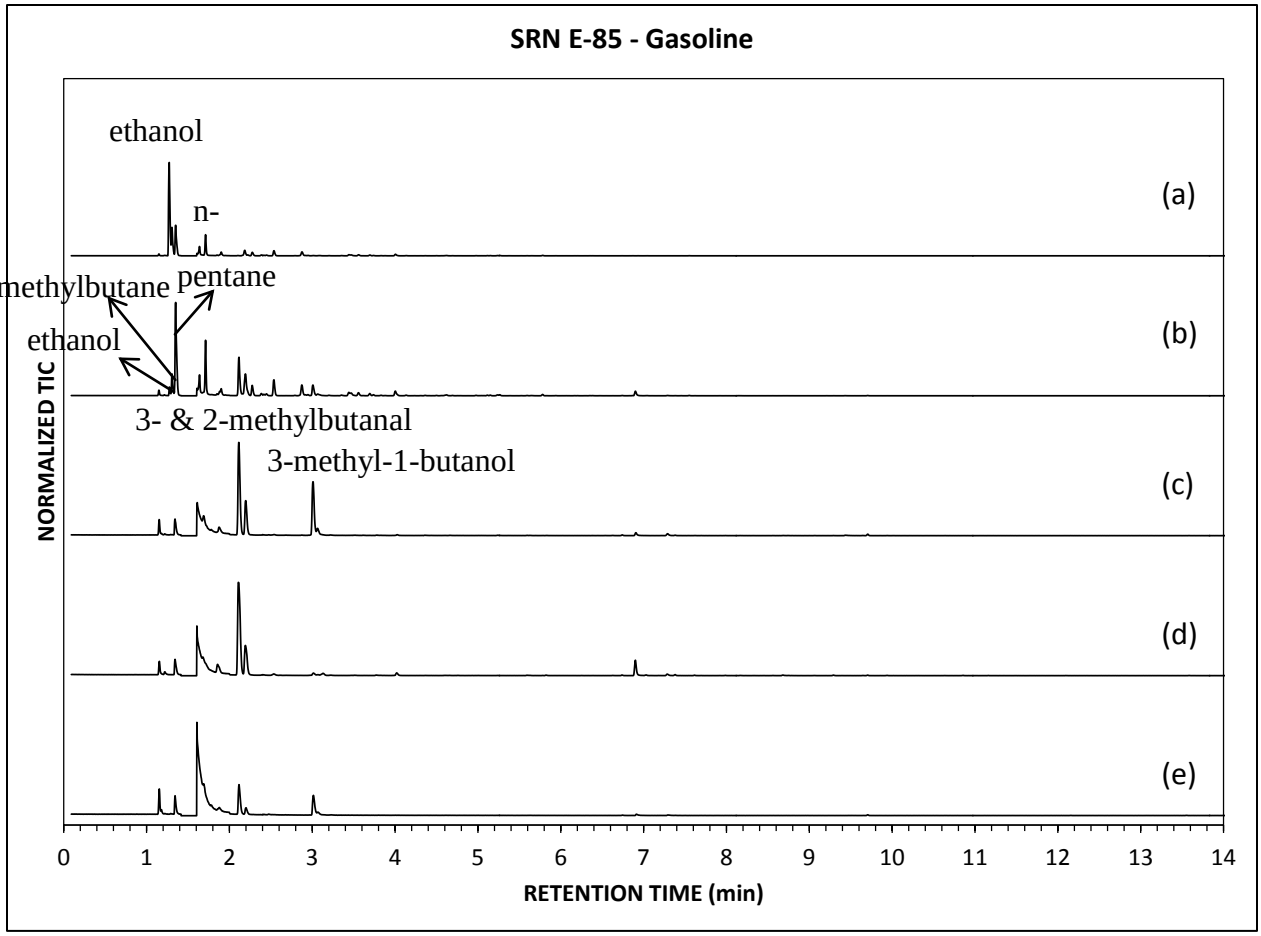

Figure B-6 Microbial degradation of gasoline, E-85: (a) 0.1% (v/v) standard, (b) Day 0, (c) Day 7, (d) Day 14, (e) and Day 21.

Appendix C Chromatograms from the BSU Soil Type and Seasonal Comparison

The soil type and seasonal comparisons not presented in Chapter 5 are presented here. For each soil type, eight sample time points were prepared in triplicate by spiking 20 μL of commercial unleaded gasoline (87 octane) onto ~100 g soil in a clean but non-sterile quart-size paint can. The samples were sealed and stored for 0, 2, 4, 7, 11, 15, 22, and 30 days. On each specified day, the samples were extracted using passive headspace adsorption-elution. One third (~7 x 9 mm^2) of a charcoal strip was placed in each can and suspended in the headspace on a pre-baked paper clip using nylon string. The re-sealed cans were heated at 85 °C for 4 h. After cooling, the charcoal strips were removed and extracted with 400 μL of pentane with vortexing for ~1 min. Samples were then analyzed by GC-MS (Agilent 6890 GC with an Agilent 5975 MSD) using a standard method for fire debris analysis, which includes a 1 μL injection volume, 20:1 split ratio, inlet temperature of 250 °C, flow rate of 1 mL/min (helium), a DB-5 30 m x 0.25 mm x 0.25 μm column, initial column temperature of 40 °C held for 2 min, temperature ramp of 20 °C/min, final temperature of 280 °C held for 3 min, solvent delay of 2 min, MS scan of 40-300 m/z, MS quad temperature of 150 °C and an MS source temperature of 230 °C.

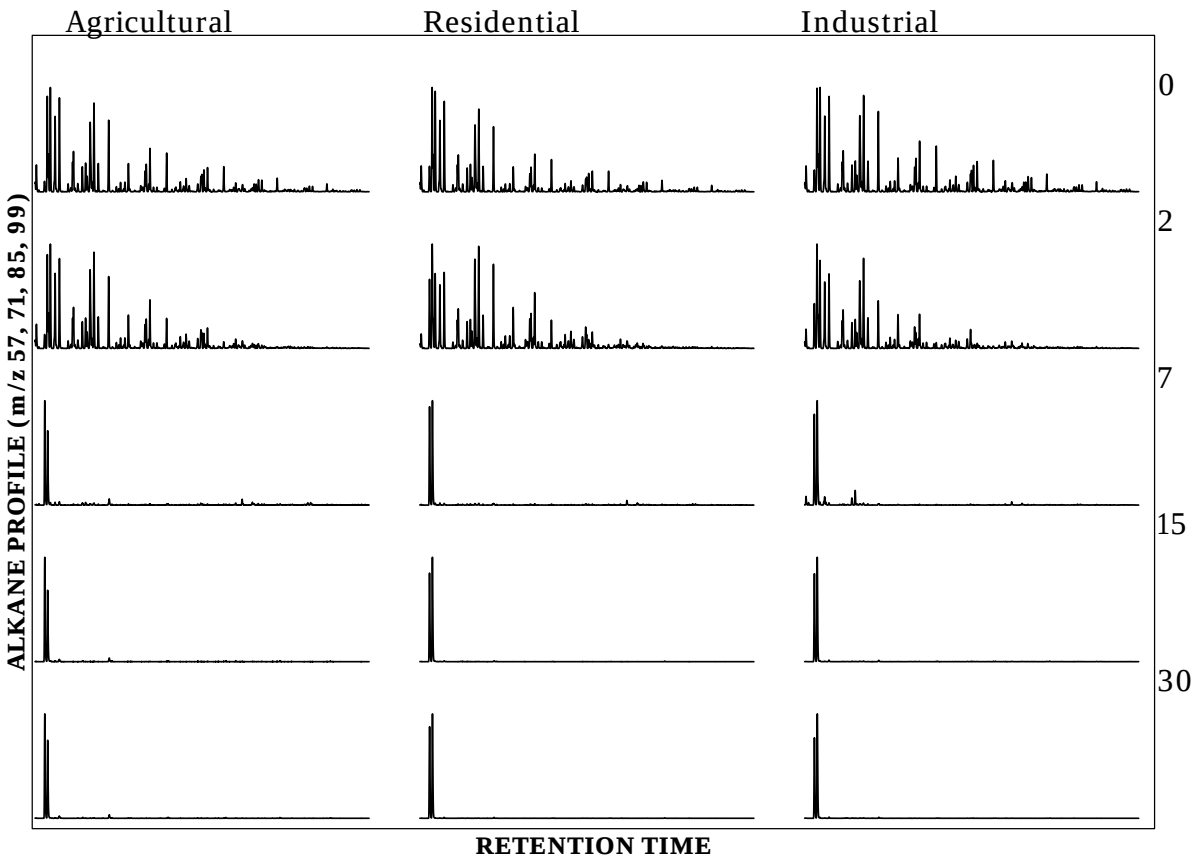

Figure C-1 Soil type comparison of the alkane profile of gasoline during the winter sampling over 30 days.

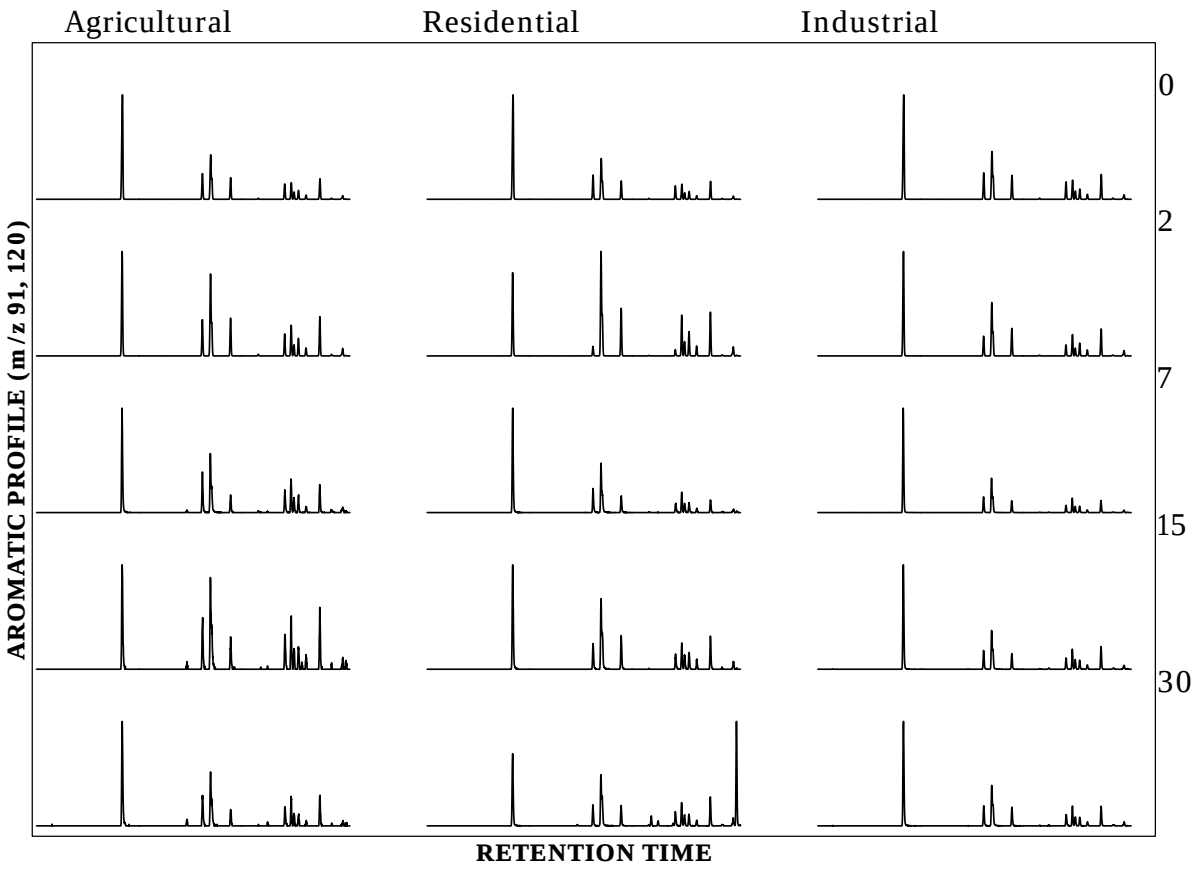

Figure C-2 Soil type comparison of the aromatic profile of gasoline during the winter sampling over 30 days.

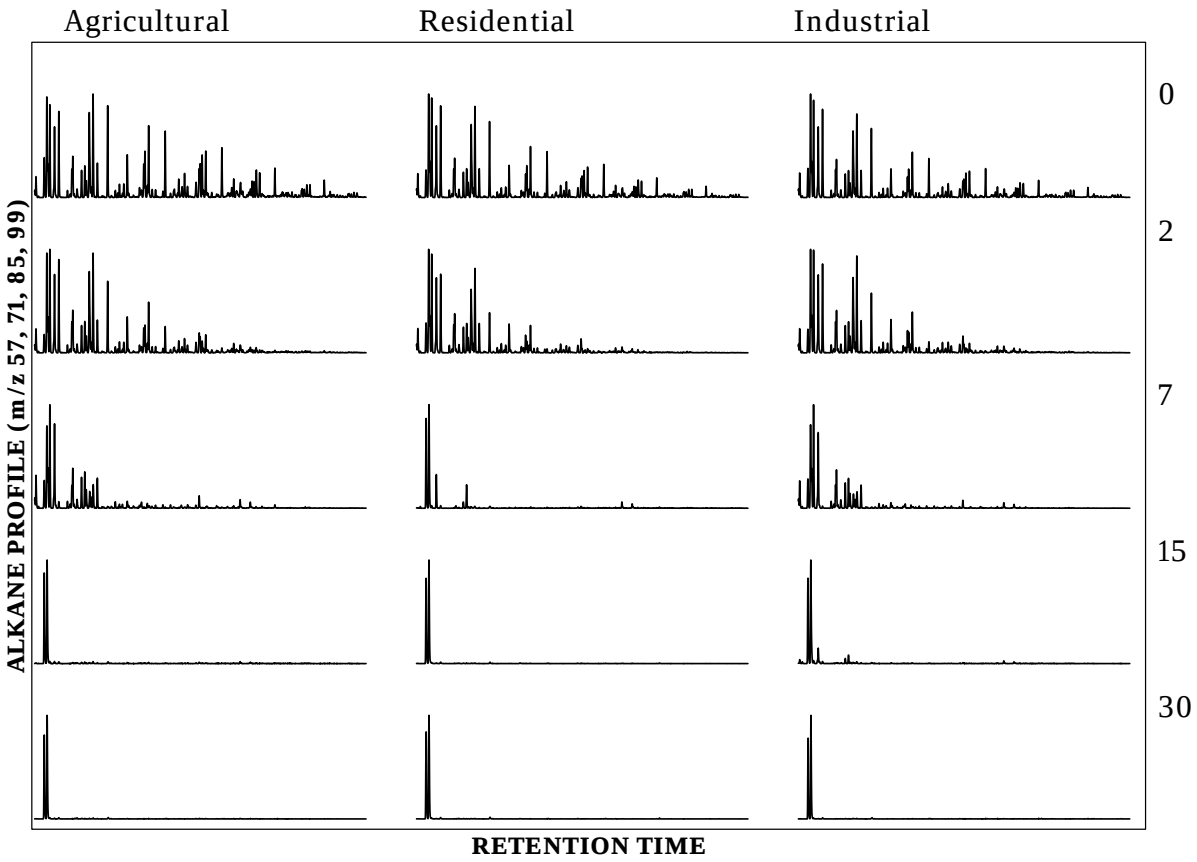

Figure C-3 Soil type comparison of the alkane profile of gasoline during the spring sampling over 30 days.

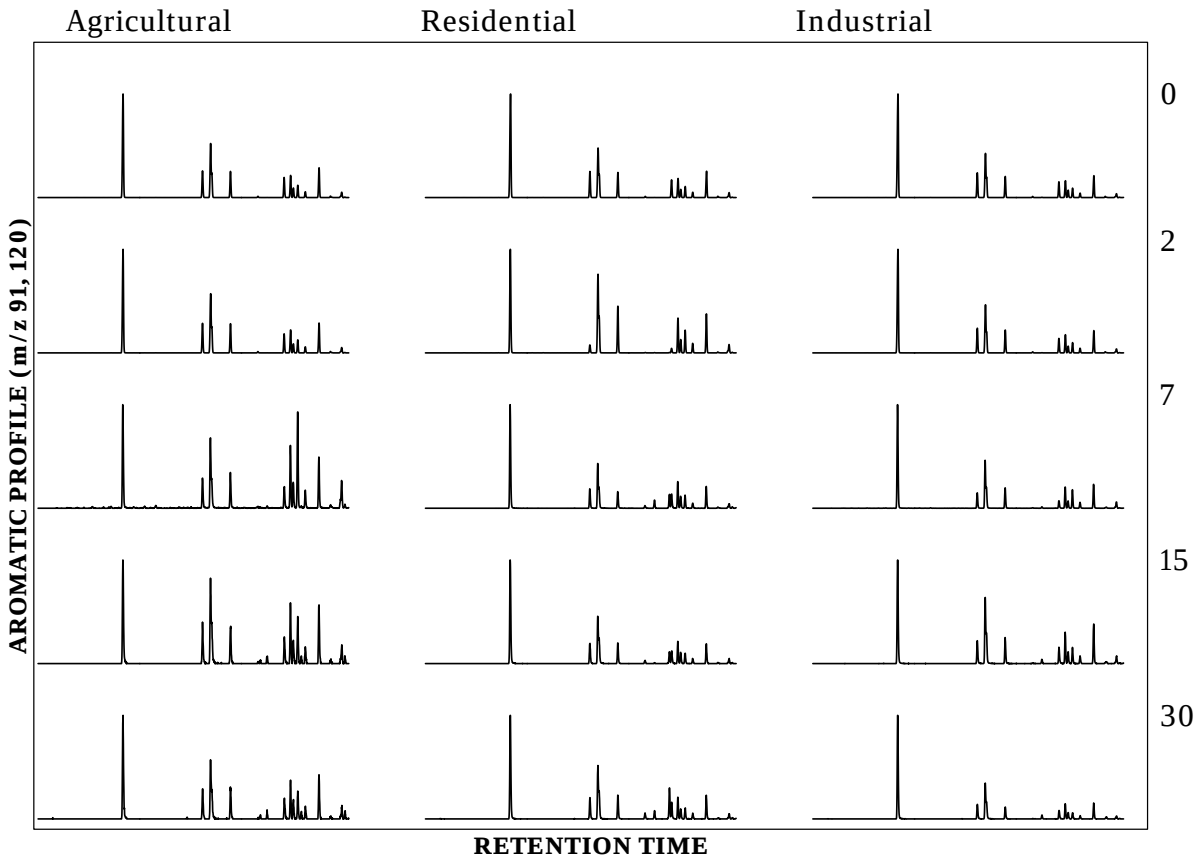

Figure C-4 Soil type comparison of the aromatic profile of gasoline during the spring sampling over 30 days.

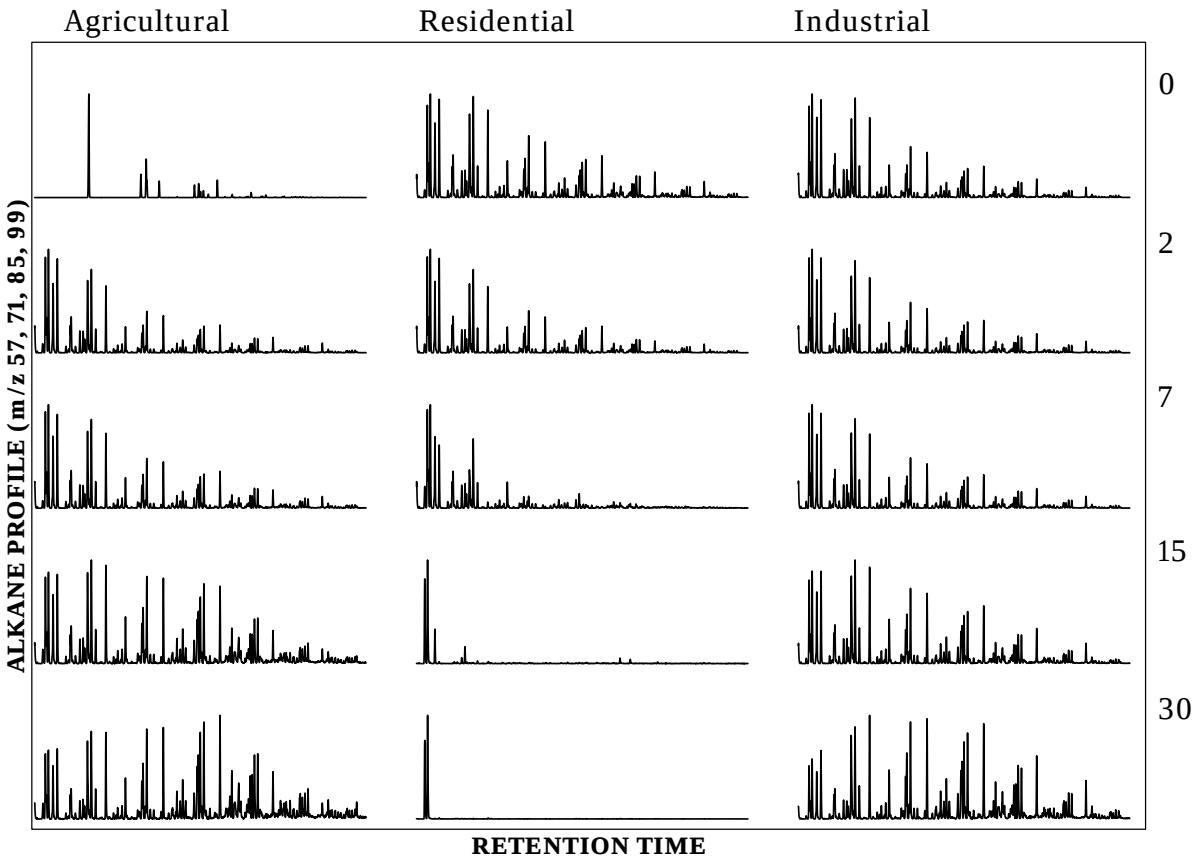

Figure C-5 Soil type comparison of the alkane profile of gasoline during the summer sampling over 30 days.

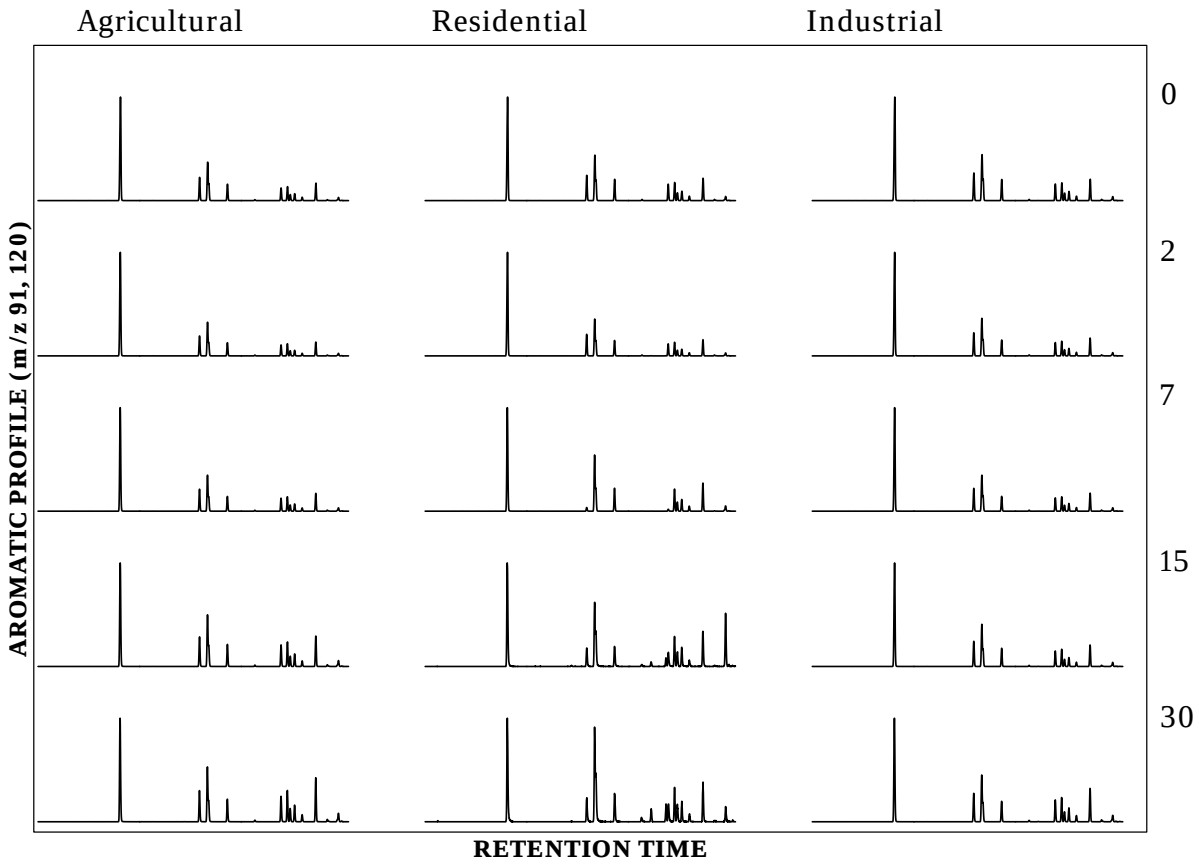

Figure C-6 Soil type comparison of the aromatic profile of gasoline during the summer sampling over 30 days.

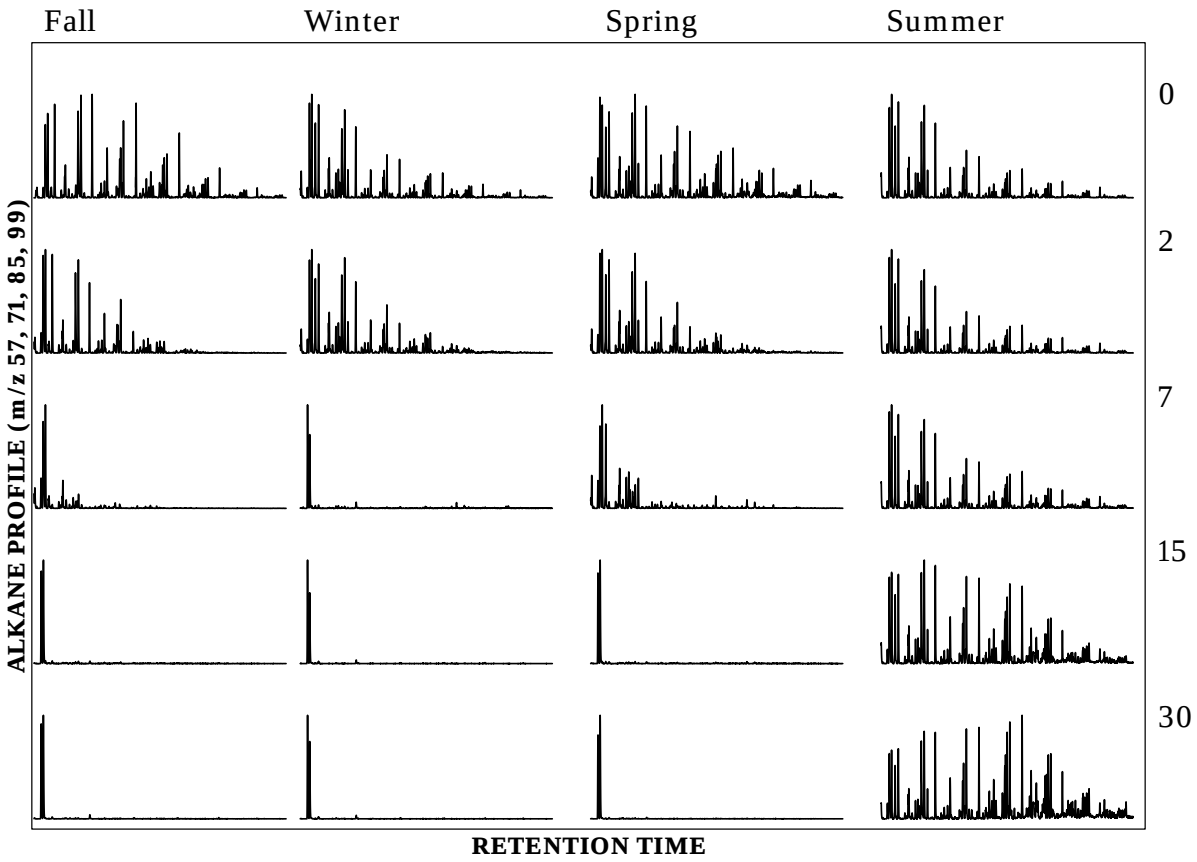

Figure C-7 Seasonal comparison of the alkane profile of gasoline in agricultural soil over 30 days.

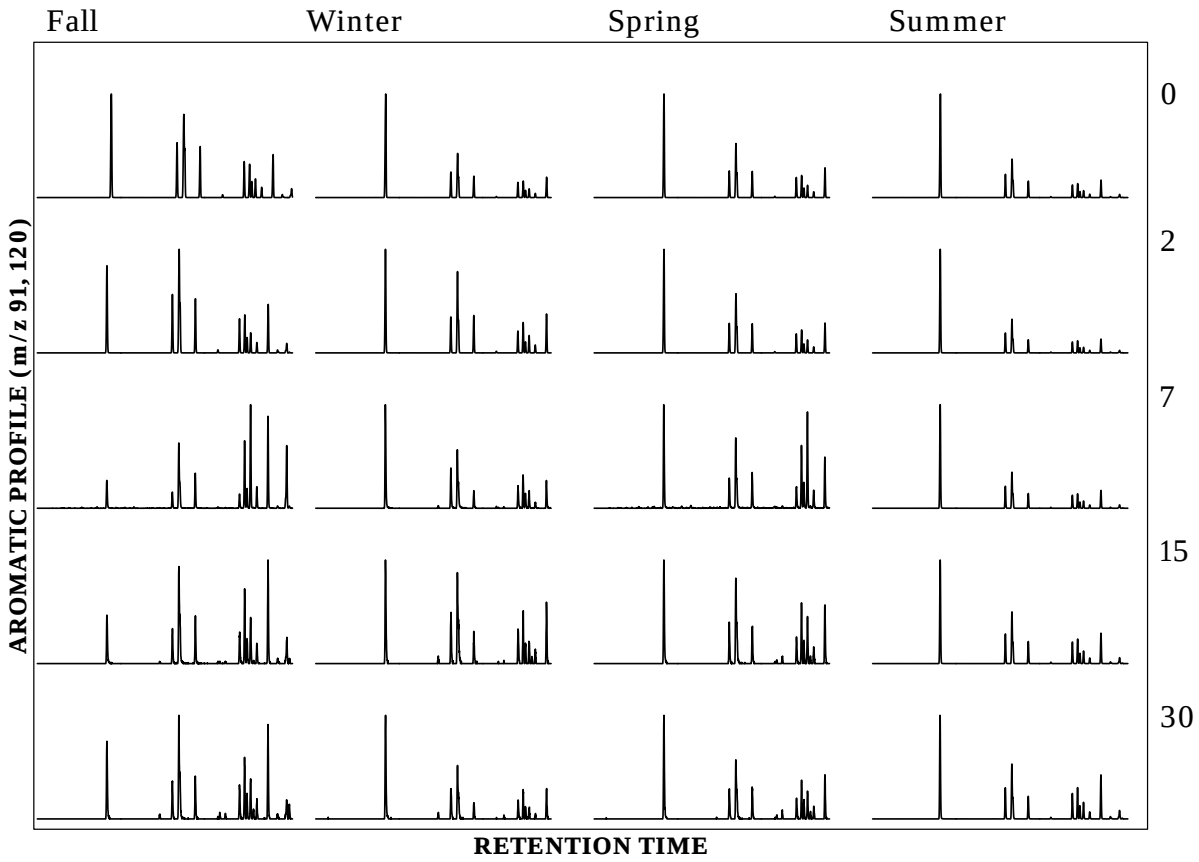

Figure C-8 Seasonal comparison of the aromatic profile of gasoline in agricultural soil over 30 days.

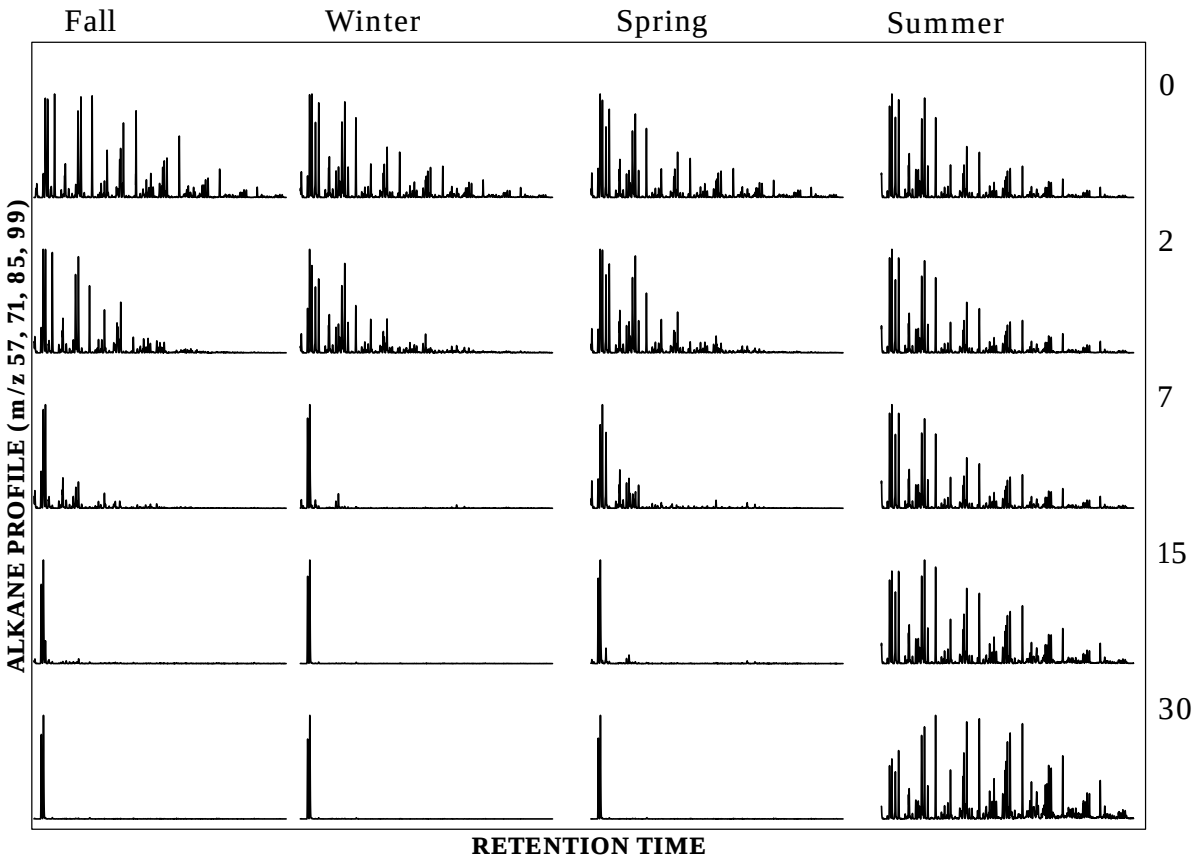

Figure C-9 Seasonal comparison of the alkane profile of gasoline in industrial soil over 30 days.

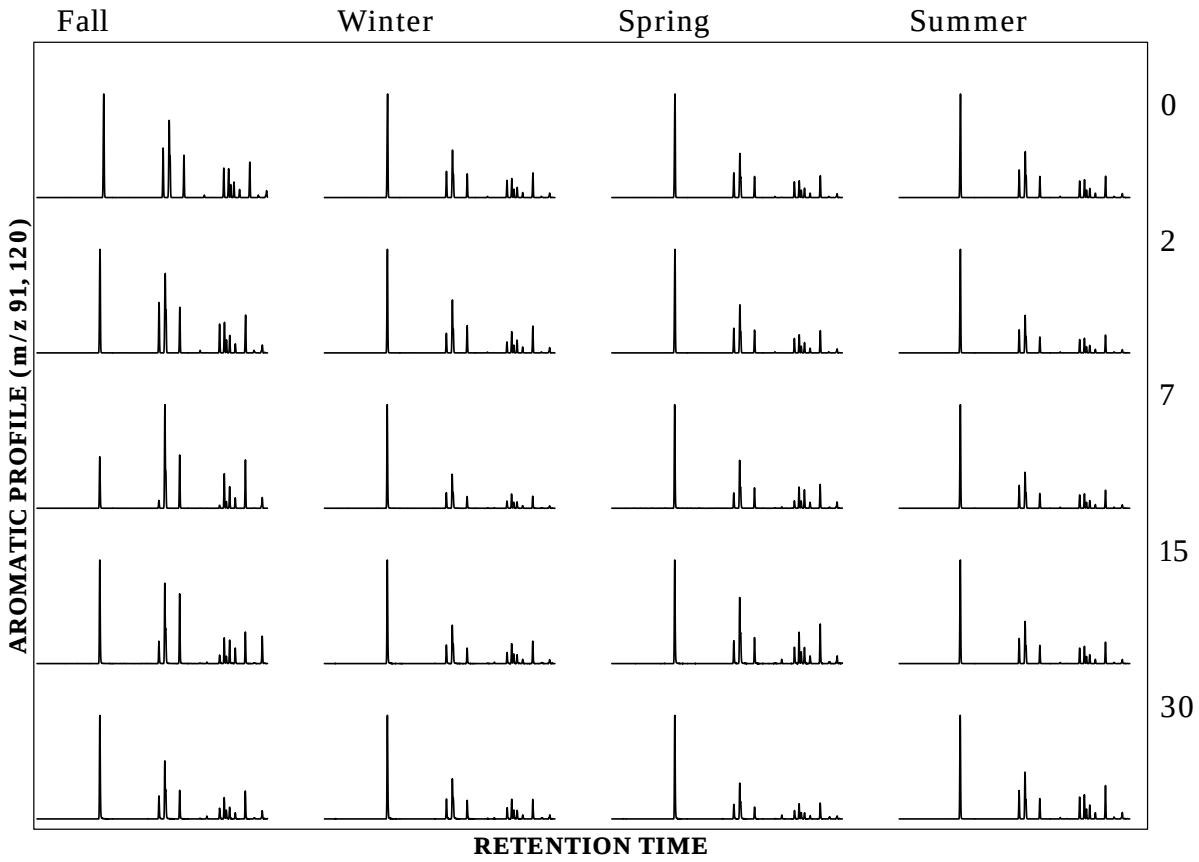

Figure C-10 Seasonal comparison of the aromatic profile of gasoline in industrial soil over 30 days.

Appendix D Chromatograms from Preservation Studies

Figures D-1 through D-3 were generated from the 2011 summer burn study using triclosan to preserve incendiary samples containing gasoline. These figures show that microbial degradation is difficult to follow and preservation is difficult to prove when the soil has not been homogenized due to the variability in the effects of weathering from the fire. Figures D-4 through D-7 were generated from the 2012 summer burn study using triclosan to preserve incendiary samples containing tiki torch fuel and diesel fuel. This summer was particularly hot and dry, which may have resulted in a significant decrease in biological activity as shown in Figures D-4 through D-7.

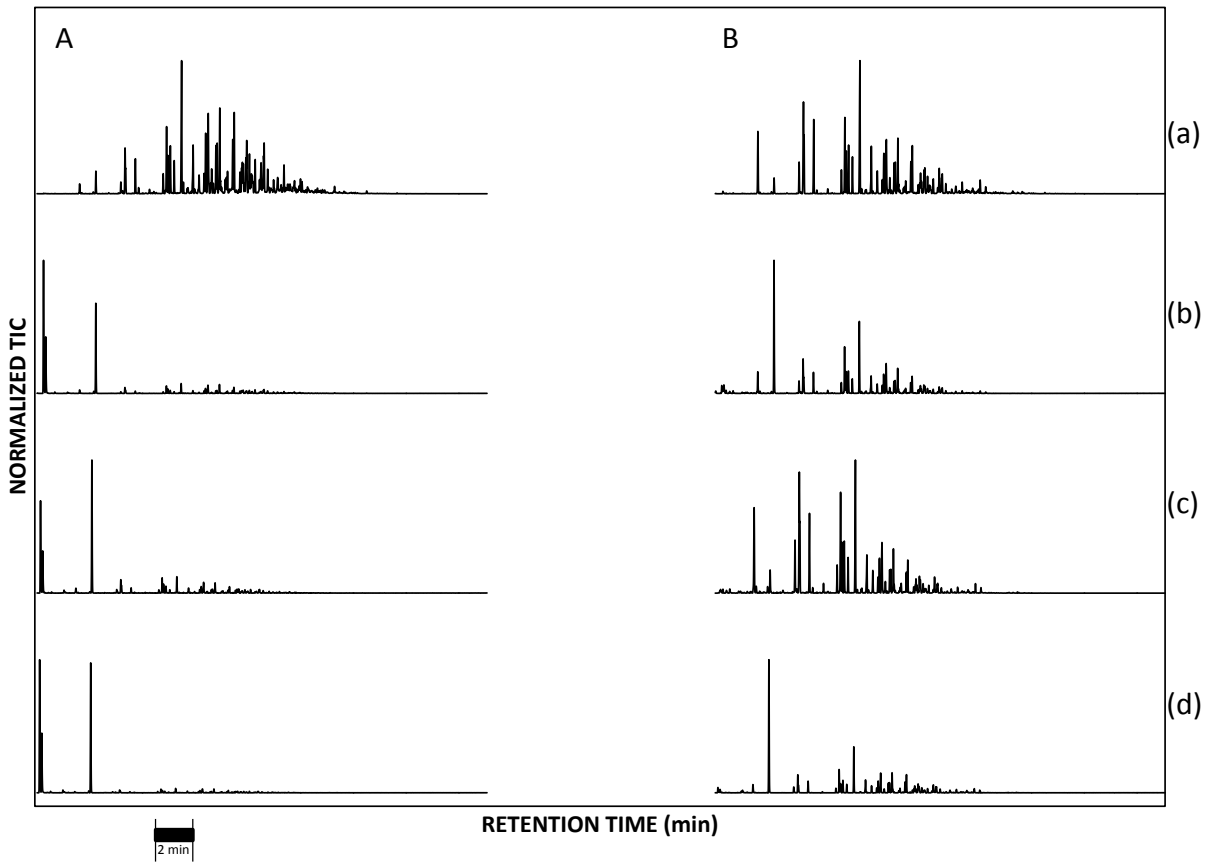

Figure D-1 TIC of gasoline for site #4 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in non-homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days.

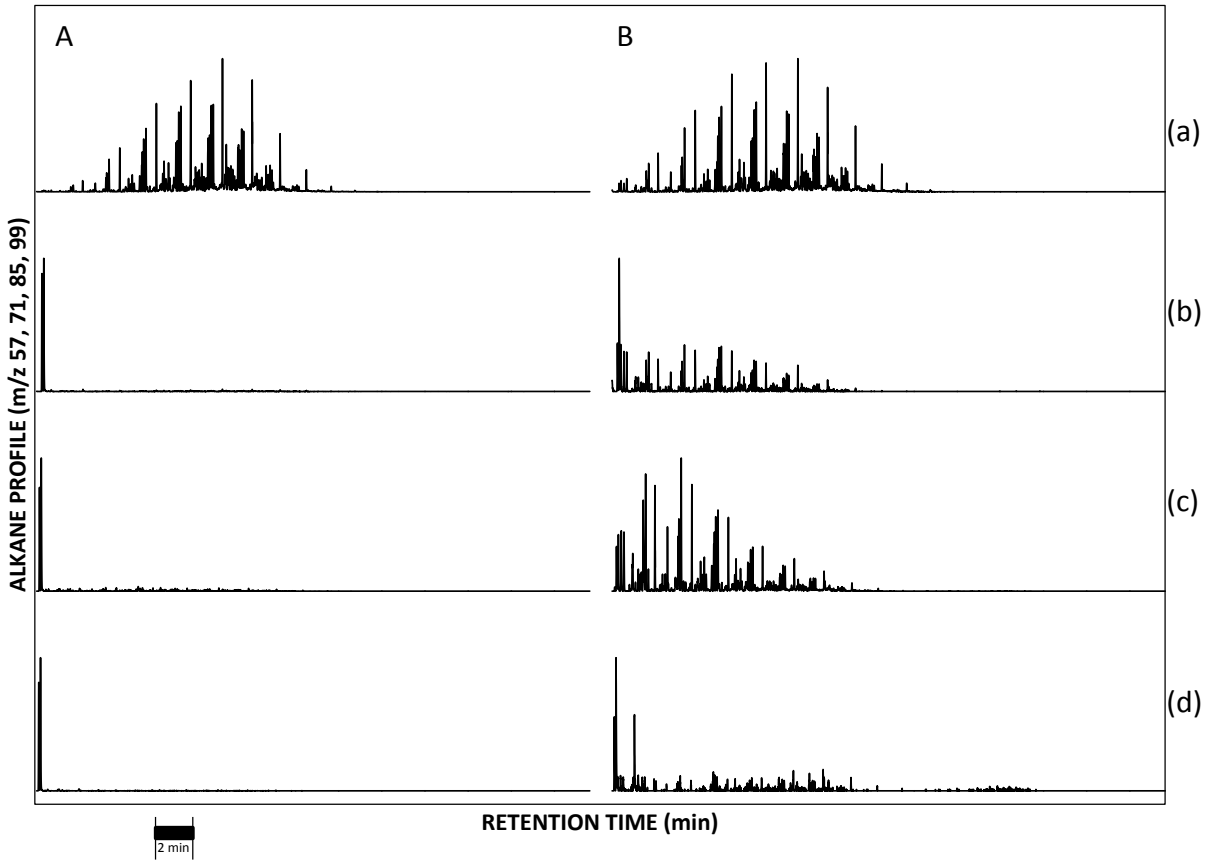

Figure D-2 Alkane profile of gasoline for site #4 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in non-homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days.

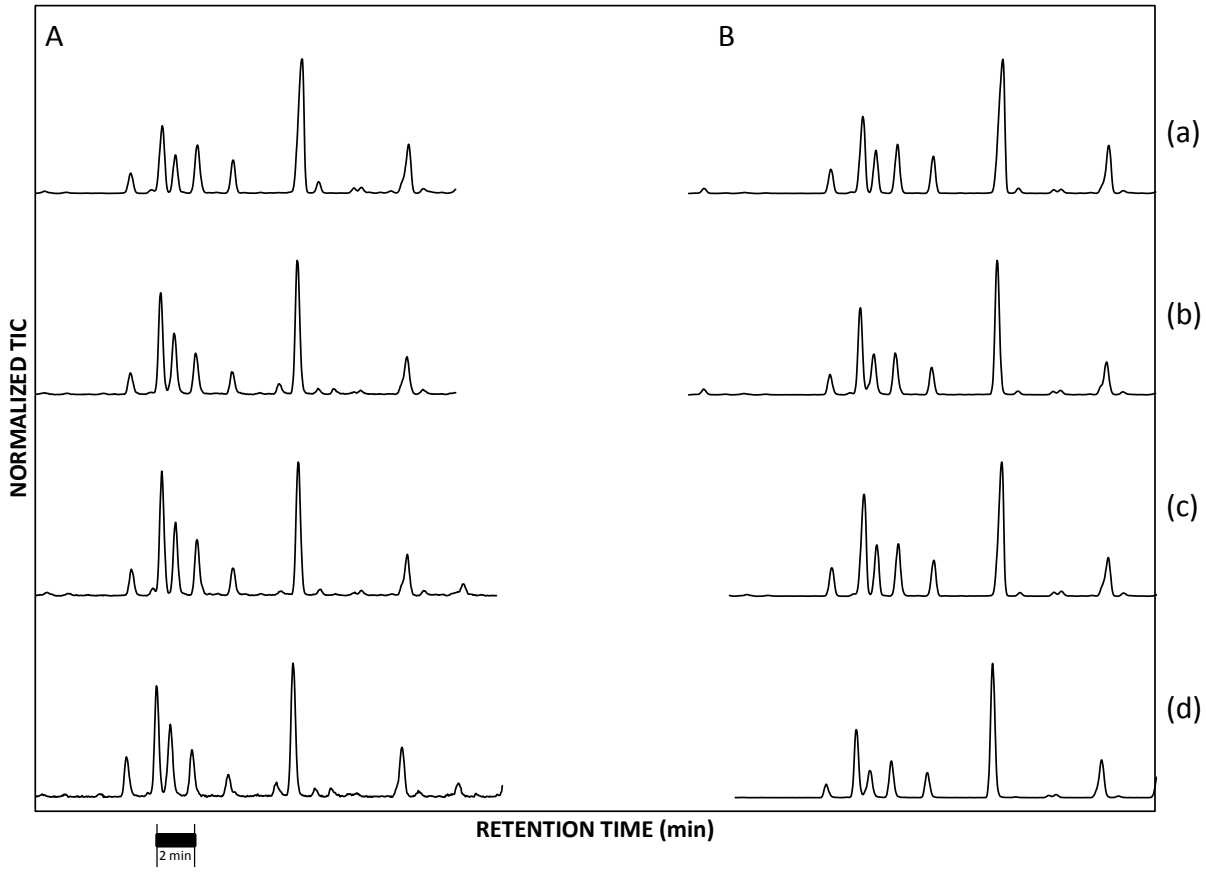

Figure D-3 C_3-alkylbenzenes in gasoline for site #4 showing microbial degradation (A) Versus preservation with 2% triclosan in 0.2M sodium hydroxide (B) in non-homogenized lawn soil over (a) 0 days, (b) 28 days, (c) 56 days, and (d) 140 days.

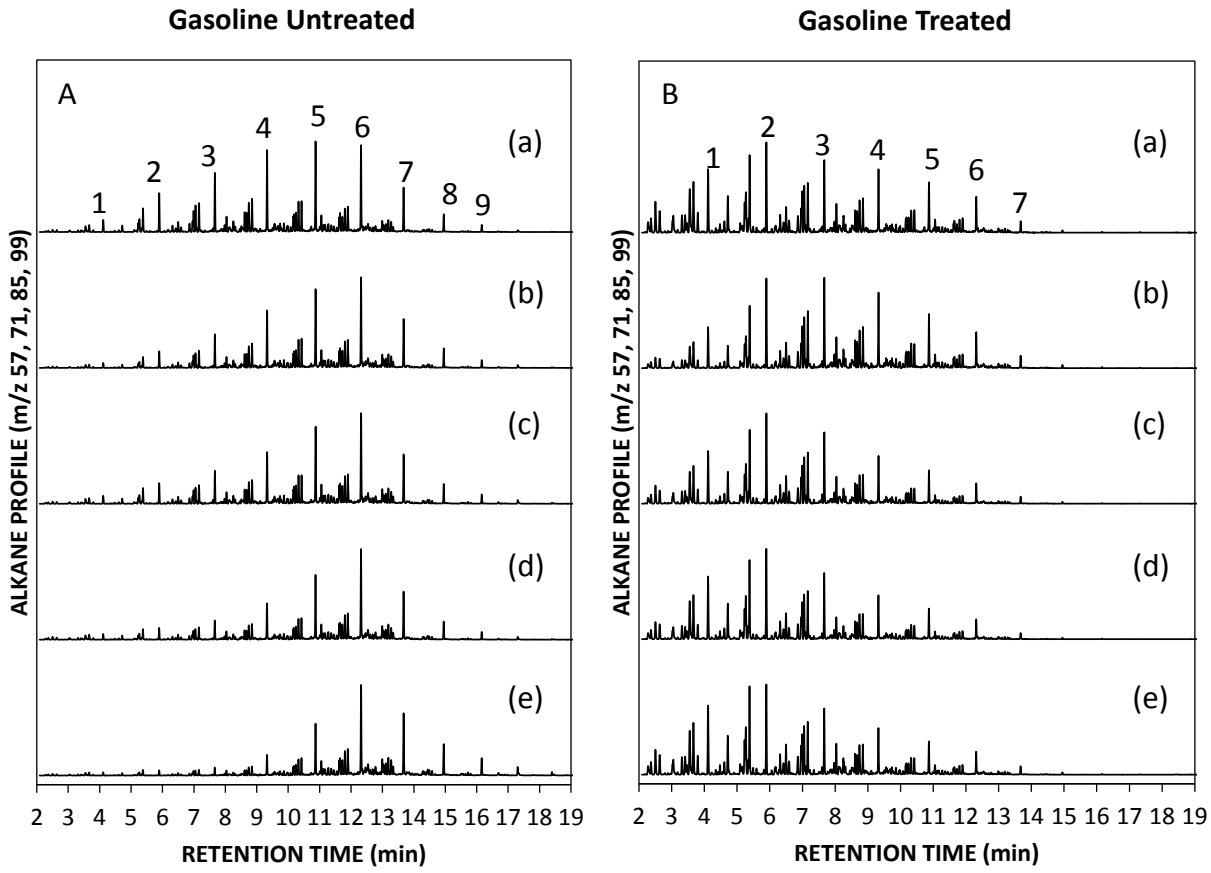

Figure D-4 Microbial degradation (A) versus preservation with 2% Triclosan in 0.2M NaOH (B) of the alkanes in gasoline from an incendiary device: (a) Day 0, (b) Day 28, (c) Day 56, (d) Day 84, and (e) Day 154. Peaks: (1) n-C_8, (2) n-C_9, (3) n-C_{10}, (4) n-C_{11}, (5) n-C_{12}, (6) n-C_{13}, (7) n-C_{14}, (8) n-C_{15}, and (9) n-C_{16}.

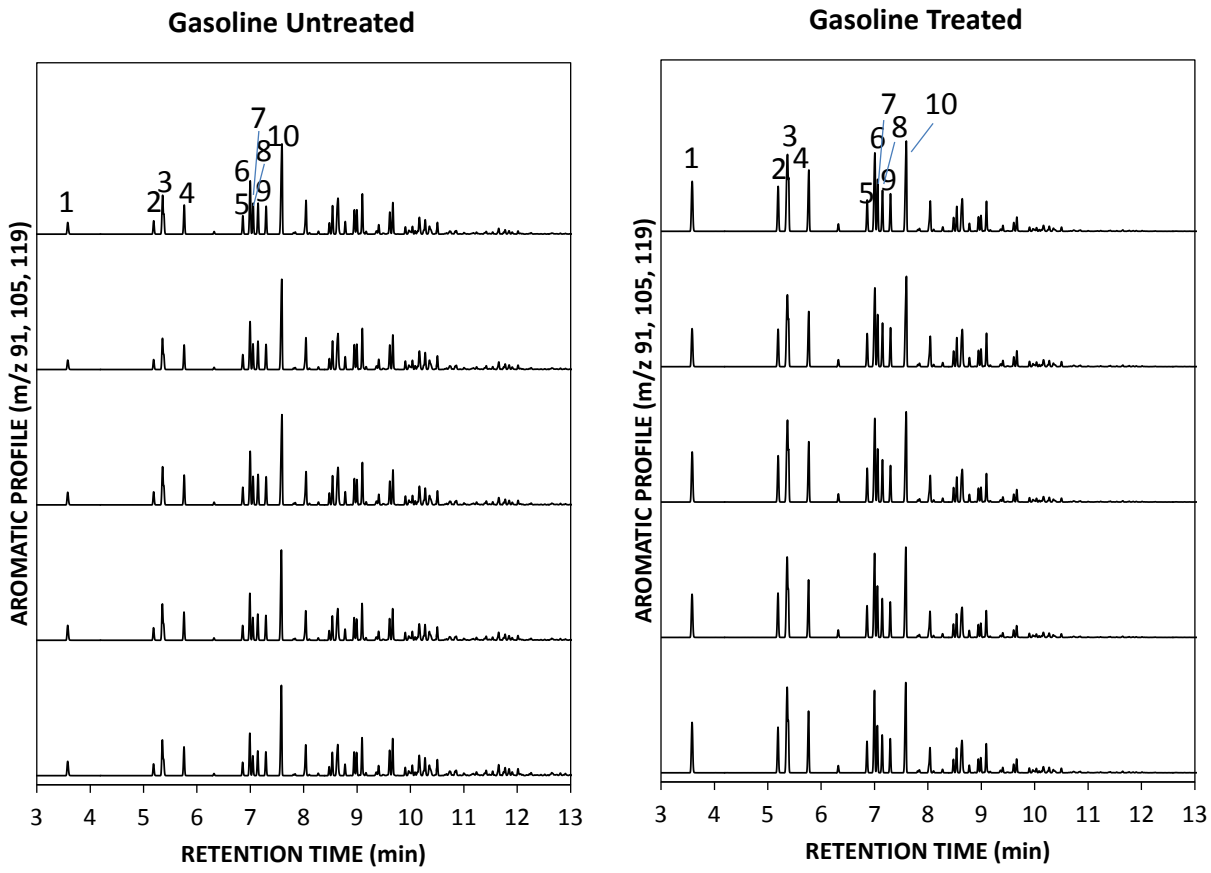

Figure D-5 Microbial degradation (A) versus preservation with 2% Triclosan in 0.2M NaOH (B) of the aromatics in gasoline from an incendiary device: (a) Day 0, (b) Day 28, (c) Day 56, (d) Day 84, and (e) Day 154. Peaks: (1) toluene, (2) ethylbenzene, (3) m- & p-xylene, (4) o-xylene, (5) propylbenzene, (6) 3-ethyltoluene, (7) 4-ethyltoluene, (8) 1,3,5-trimethylbenzene, (9) 2-ethyltoluene, and (10) 1,2,4-trimethylbezene.

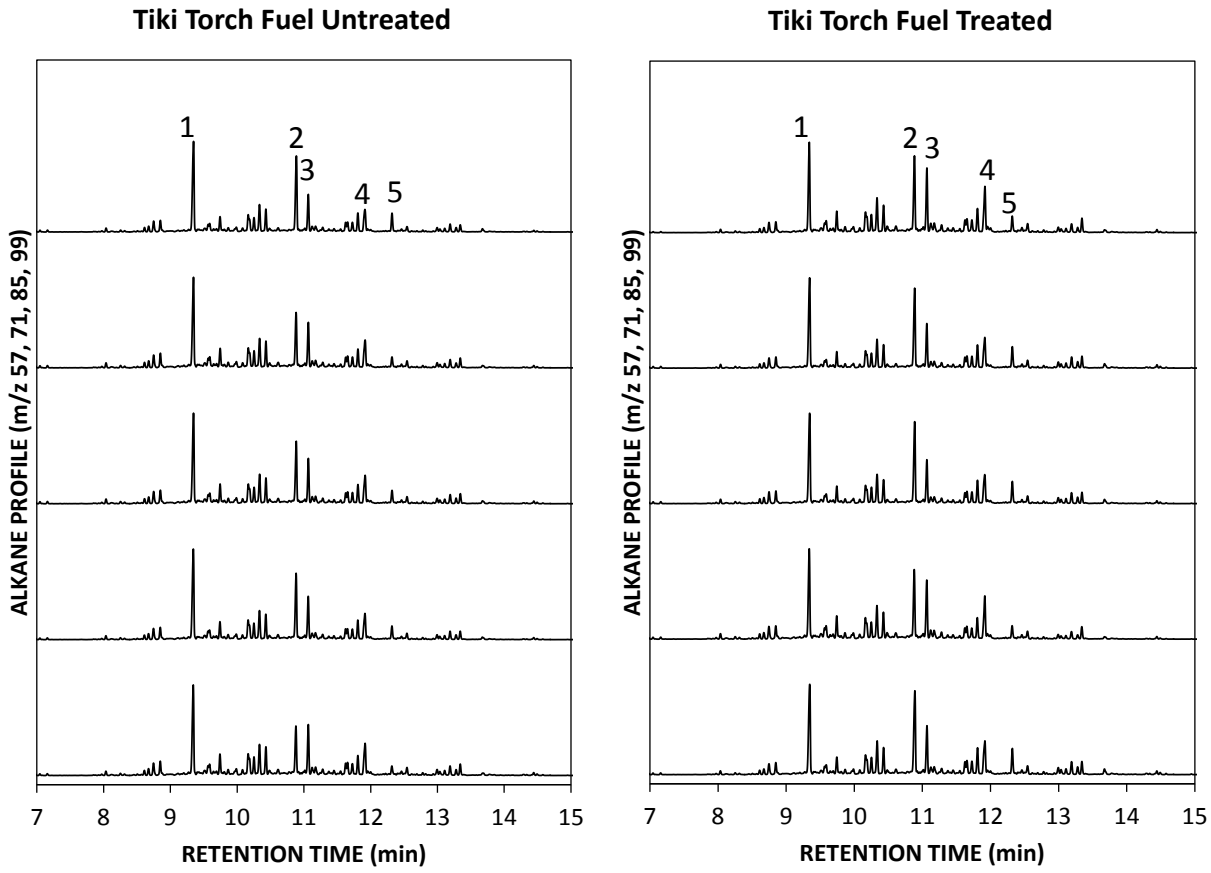

Figure D-6 Microbial degradation (A) versus preservation with 2% Triclosan in 0.2M NaOH (B) of the alkanes in a Tiki torch fuel from an incendiary device: (a) Day 0, (b) Day 28, (c) Day 56, (d) Day 84, and (e) Day 154. (1) n-C_{11}, (2) n-C_{12}, (3) 2,6-dimethylundecane, (4) 4,6-dimethyldodecane, (5) n-C_{13}.

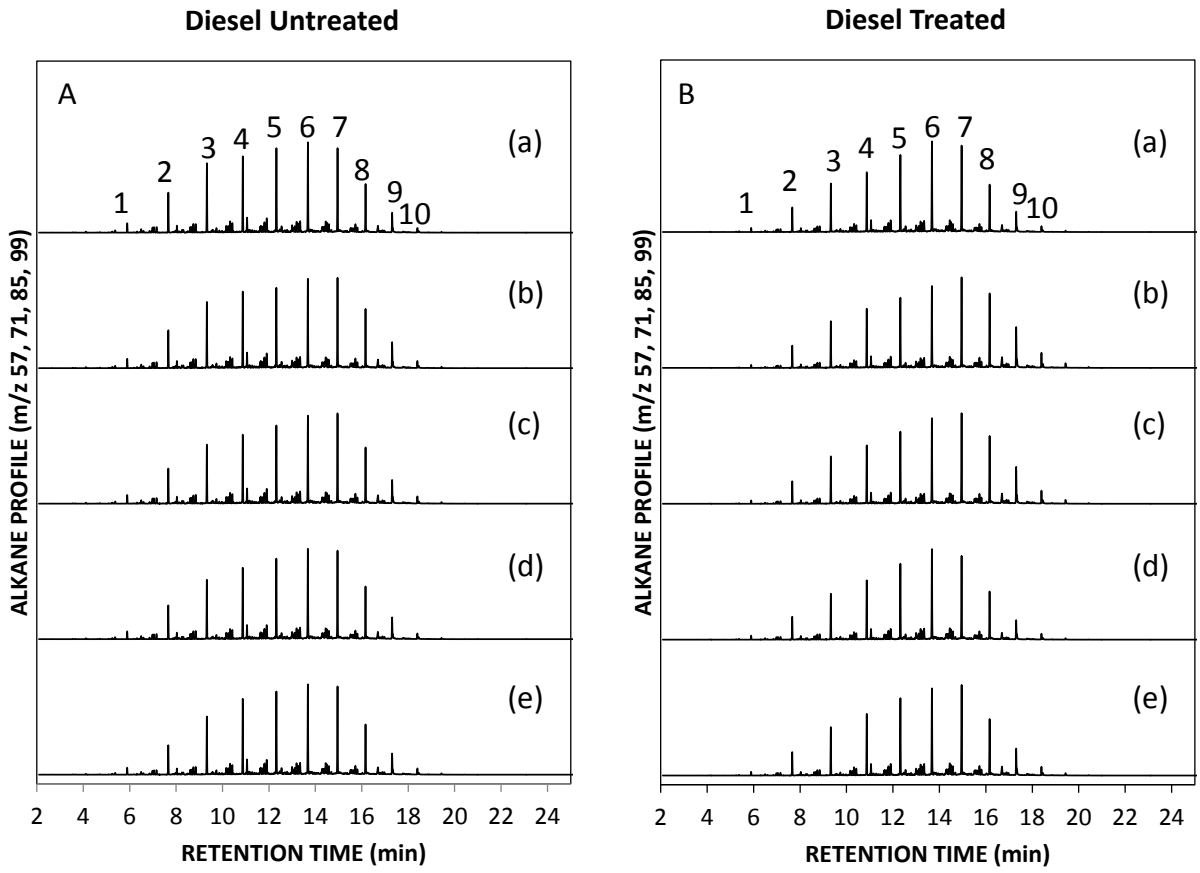

Figure D-7 Microbial degradation (A) versus preservation with 2% Triclosan in 0.2M NaOH (B) of the alkanes in a diesel fuel from an incendiary device: (a) Day 0, (b) Day 28, (c) Day 56, (d) Day 84, and (e) Day 154. Peaks: (1) n-C_9, (2) n-C_{10}, (3) n-C11, (4) n-C12, (5) n-C_{13}, (6) n-C_{14}, (7) n-C_{15}, (8) n-C_{16}, (9) n-C_{17}, and (10) n-C_{18}.

VITA

VITA

Dee Ann Turner
Graduate School, Purdue University

Education

B.S., Chemistry, 2008, Purdue University, Indianapolis, Indiana
Ph.D., Analytical Chemistry, 2013, Purdue University, West Lafayette, Indiana

Publications

Turner, D. A. and Goodpaster, J. V., The Effects of Season and Soil Type on Microbial Degradation of Gasoline Residues from Incendiary Devices, (2013) *Analytical and Bioanalytical Chemistry*, *405*(5): *1593*-1599.

Turner, D. A. and Goodpaster, J. V., Comparing the Effects of Weathering and Microbial Degradation on Gasoline Using Principal Components Analysis, (2012) *Journal of Forensic Sciences,* 57(1): 64-69.

Turner, D. A. and Goodpaster, J. V., The Effect of Microbial Degradation on the Chromatographic Profiles of Tiki Torch Fuel, Lamp Oil, and Turpentine, (2011) *Journal of Forensic Sciences,* 56(4): 984-987.

Turner, D. A. and Goodpaster, J. V., The Effects of Microbial Degradation on Ignitable Liquids, (2009) *Analytical and Bioanalytical Chemistry*, 394(1): 363-371.

Presentations

D.A. Turner, J.V. Goodpaster, M.R. Williams, and M.E. Sigman. "Trends in Microbial Degradation of Various Ignitable Liquids in Soil," *National Meeting of the American Chemical Society,* Indianapolis, IN 09/10/13.

D.A. Turner, and J.V. Goodpaster. "A Comprehensive Study of Weathering and Microbial Degradation of Ignitable Liquids," *Annual Meeting of the American Academy of Forensic Sciences,* Washington, DC 02/22/13.

D.A. Turner, and J.V. Goodpaster. “Microbial Degradation of Gasoline Used in Incendiary Devices: Triclosan as a Solution,” *National Meeting of the American Chemical Society*, San Diego, CA 03/25/12 and 03/26/12.

D.A. Turner, and J.V. Goodpaster. “Microbial Degradation of Gasoline Used in Incendiary Devices: Triclosan as a Solution,” *PITTCON Conference and Expo*, Orlando, FL 03/14/12.

D.A. Turner, and J.V. Goodpaster. “The Effect of Season and Soil Type on the Microbial Degradation of Gasoline,” *Annual Meeting of the American Academy of Forensic Sciences,* Atlanta, GA 02/25/12.

S. Bawel, D.A. Turner, and J.V. Goodpaster. “Effectiveness of Antibiotics and Household Products as an Antimicrobial Solution in Fire Debris Samples,” *Midwestern Association of Forensic Scientists,* Lombard, IL 09/22/11.

D.A. Turner, S. Bawel, and J.V. Goodpaster. “Effectiveness of Triclosan as an Antimicrobial Agent for Fire Debris Samples,” *Midwestern Association of Forensic Scientists,* Lombard, IL 09/22/11.

D.A. Turner and J.V. Goodpaster. “Development of a Sampling System to Stabilize Ignitable Liquid Residues in Fire Debris,” *American Society of Crime Laboratory Directors*, Denver, CO 09/21/11.

D.A.Turner, A. Flores, and J.V. Goodpaster. “Monitoring and Characterizing Microbial Degradation of Gasoline on Different Soil Types,” *Central Regional Meeting of the American Chemical Society,* Indianapolis, IN 6/10/11.

D.A.Turner and J.V. Goodpaster. “Die Criters Die: The Challenges of Mitigating Microbial Degradation in Fire Debris Samples,” *Young Forensic Scientists Forum Bring Your Own Slides* at the *Annual Meeting of the American Academy of Forensic Sciences,* Chicago, IL 2/23/11.

D.A.Turner, J.V. Goodpaster, and L. Lang. “Effects of Temperature, Exposure Time, and Sample Size on the Recovery of Smokeless Powder Constituents from Post-burn Samples,” *Annual Meeting of the American Academy of Forensic Sciences*, Chicago, IL 2/24/11.

A. Flores, D.A. Turner, and J.V. Goodpaster. “Preventing Ignitable Liquid Degradation Using Antimicrobial Agents,” *Annual Meeting of the American Academy of Forensic Sciences*, Chicago, IL 2/24/11.

D,A, Turner, V.H. Herring, and J.V. Goodpaster, “Chemical Agents for Use in Preserving Fire Debris Evidence Against Microbial Degradation,” *Annual Meeting of the American Academy of Forensic Sciences*, Seattle, WA 2/25/10.

D.A. Turner, J.V. Goodpaster, “Multivariate Statistical Analysis of the Effects of Weathering and Microbial Degradation of Ignitable Liquids Used in Incendiary Devices,” *Joint Forensic Association Meeting*, Orlando, FL 10/22/09.

D.A. Turner, J.V. Goodpaster, “The Effect of Microbial Degradation on Ignitable Liquids,” *Annual Meeting of the American Academy of Forensic Sciences*, Denver, CO 2/20/09.

D.A. Turner, J.V. Goodpaster, “The Effects of Microbial Degradation on Ignitable Liquids,” *Annual Meeting of the Midwestern Association of Forensic Scientists*, Des Moines, IA 10/2/08.